ACQUIRED CARBAPENEMASES IN GRAM-NEGATIVE BACILLI IN SINGAPORE

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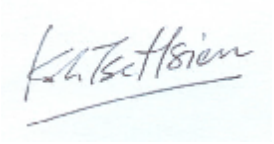
DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety.

I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.



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Summary

The recent global spread of carbapenemase genes is an emerging problem. The aim of this thesis was to identify the reasons behind the increase in carbapenem-resistance in Gram-negative bacilli in Singapore. While multiple factors probably contribute to this emergence in Singapore, this thesis hypothesised that the introduction of global clones of carbapenemase-producing strains with the potential to cause outbreaks is an important reason. This hypothesis was tested in *Pseudomonas aeruginosa, Acinetobacter baumannii,* and *Enterobacteriaceae.*

The first layer of molecular typing used a discriminatory fingerprinting method to determine the presence of clones within the local population of isolates. From the fingerprinting data, representative isolates were selected for a second layer of typing using MLST and AFLP™.

Major findings regarding acquired carbapenemases in *P. aeruginosa*

In 2000, 21 of 2,094 non-duplicate *P. aeruginosa* had metallo-β-lactamase (MBL) genes. With the exception of 1 isolate with $bla_{\text{IMP-7}}$, all other isolates had $bla_{\text{IMP-1}}$ and belonged to one of two clones. These 2 clones were not international outbreak clones and did not emerge dominant in 2008. Of 2,552 non-duplicate *P. aeruginosa* isolated in 2008, 9 yielded a product by multiplex PCR for MBL genes. The 2008 isolates were quite heterogenous with $bla_{\text{VIM-2}}$ and $bla_{\text{VIM-6}}$ emerging in *P. aeruginosa* for the first time in Singapore.

International clones belonging to CC235 appeared in 2008 and contained a mix of MBL genes.

Major findings regarding acquired carbapenemases in the *Acinetobacter calcoaceticus-Acinetobacter baumannii* species complex (Acb)

One hundred and ninety-three non-duplicate Acb were collected over a 1-month period from 6 hospitals in 2006. A high proportion of Acb were resistant to carbapenems (63.7%). Most carbapenem-resistant *A. baumannii* and *Acinetobacter nosocomialis* possessed the $bla_{\text{OXA-23-like}}$ gene. Most carbapenem-resistant *Acinetobacter pittii* had the $bla_{\text{OXA-58-like}}$ gene. In an earlier study carried out in 1 hospital, 7.7% of Acb collected in 1996, and 21.2% of Acb collected in 2001, were resistant to carbapenems. The main outbreak isolates of carbapenemase-producing *A. baumannii* in 1996 did not belong to international outbreak clones. Outbreak isolates from 2001 belonged to European clones I and II. One of these clones could still be found among isolates collected in 2006.

Major findings regarding acquired carbapenemases in *Enterobacteriaceae*

Since the first isolate of carbapenemase-producing *Enterobacteriaceae* in Singapore in 1996, we have collected a further 40 isolates. The only large outbreak clone was $bla_{\text{OXA-181}}$-positive *Klebsiella pneumoniae* belonging to ST14. Even though *K. pneumoniae* with $bla_{\text{KPC-2}}$ were not clonal by PFGE, they all belonged to ST11.

Global clones are present among carbapenemase-producing *P. aeruginosa*, *A. baumannii*, and *Enterobacteriaceae* in Singapore. They are likely to be responsible for the increase in carbapenem-resistant *A. baumannii* but not *P. aeruginosa*. However, the global clones of carbapenemase-producing *P. aeruginosa* and *Enterobacteriaceae* are recently introduced and the situation may change in the future. These findings demonstrate the diversity of acquired carbapenemase genes in Singapore, their distribution among different species, and suggest the mechanisms underlying their spread both locally as well as internationally.

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List of Abbreviations

Acb	*Acinetobacter calcoaceticus-Acinetobacter baumannii* species complex
AFLP™	amplified fragment length polymorphism
AMK	amikacin
BO	boronic acid
c.a.	approximately
CAZ	ceftazidime
CC	clonal complex
CIP	ciprofloxacin
CRE	carbapenemase-producing *Enterobacteriaceae*
CX	cloxacillin
DNA	deoxyribonucleic acid
DP	dipicolinic acid
EDTA	ethylenediaminetetraacetic acid
ESBL	extended spectrum β-lactamase
FEP	cefepime
GEN	gentamicin
h	hour
HCl	hydrochloride
ICE	integrative conjugative element
IPM	imipenem
IS	insertion sequence
IS*CR*s	insertion sequence with common regions

ITS	intergenic spacer
LB	Luria-Bertani
MBL	metallo-β-lactamase
MEM	meropenem
MIC	minimal inhibitory concentration
min	minute
MLST	multilocus sequence typing
O.D.	optical density
pAmpC	plasmid AmpC β-lactamase
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
POL	polymixin B
rpm	revolutions per minutre
rRNA	ribosomal ribonucleic acid
s	second
SAM	sulbactam-ampicillin
ST	sequence type
TBE	tris/Borate/EDTA
TE	tris/EDTA
TGC	tigecycline
TZP	piperacillin-tazobactam

Chapter 1 Introduction and Literature Review

The β-lactam antibiotics

The discovery of penicillin by Alexander Fleming in 1928, its purification by Florey and others at the Sir William Dunn School of Pathology in Oxford, and the success of mass production methods in corporate North America have changed our perspective of infectious diseases forever (1). While other antibacterial chemicals like the sulphonamides already existed, penicillin had much greater potency against many important bacterial pathogens and had relatively minor side effects apart from the occasional hypersensitivity reaction.

Introduced into large scale use during the Second World War, penicillin was able to treat infected wounds and burns, thereby saving countless lives that would otherwise have succumbed to gas gangrene and the streptococcal infections that had plagued previous generations of injured soldiers. Away from the battlefield, venereal diseases like gonorrhoea and syphilis that often accompany warfare were also highly susceptible to the new drug. Once the war ended, penicillin found utility treating pneumonia and meningitis, roles it continues to perform to this day.

The first cephalosporin was discovered by Giuseppe Brotzu in 1945 (2). Like Fleming before him, Brotzu lacked the ability to extract and purify his

antibiotic. This was left to scientists at the aforementioned Sir William Dunn School of Pathology. In 1953, cephalosporin C was discovered by Newton and Abraham (3). Together with penicillin, these proved to be the forerunners of a most versatile family of compounds with potent and specific antibacterial activity.

Both the penicillins and the cephalosporins have a β-lactam ring. In the case of penicillin, this is fused to a 5-membered thiazolidine ring and in the case of the cephalosporins a six-membered dihydrothiazine ring. Modifications of the basic structure by adding different side chains resulted in derivative drugs with new properties. The poor activity against Gram-negative bacilli that was a feature of the original penicillin was overcome by adding an amino group to give ampicillin (4). However as early as 1940, an enzyme had been discovered that was able to hydrolyze the β-lactam ring of penicillin (5). This was to be but the first of a multitude known collectively as β-lactamases. These enzymes are related at a structural and functional level to the penicillin-binding proteins involved in bacterial cell wall synthesis (6). β-lactams may be substrates or inhibitors for both β-lactamases and penicillin binding proteins. The difference is that β-lactamases hydrolyze β-lactams at a much higher rate than penicillin binding proteins (7).

The first β-lactamases were essentially penicillinases found in staphylococci (8) and *Bacillus* spp. (9). In the latter part of the 1970s, β-lactamases with the ability to hydrolyze broader spectrum β-lactams including ampicillin began to

spread. This included the TEM-1, SHV-1, and OXA-1 plasmid-borne β-lactamases found in many *Enterobacteriaceae* (10). Pharmaceutical companies responded with cephalosporins like cefuroxime, the first to demonstrate stability to the new β-lactamases (11). This was followed by oxyimino-cephalosporins like cefotaxime (12) and ceftazidime (13) with even more potent activity against Gram-negative bacilli. However as the use of these extended-spectrum cephalosporins became more widespread, it promoted the emergence of a corresponding generation of extended-spectrum β-lactamases (ESBLs). Most of these were point mutations of the aforementioned TEM, SHV and OXA plasmid-borne β-lactamases. In addition, many Gram-negative bacilli also intrinsically produced a chromosomal cephalosporinase (AmpC) able to hydrolyze extended-spectrum cephalosporins. These enzymes are usually not produced unless induced but in certain species like *Enterobacter cloacae* and *Citrobacter freundii*, mutations may occur in the regulatory genes with high frequency allowing the enzymes to be constitutively hyper-produced in large quantities. Cephalosporinases have also mobilized onto plasmids (pAmpC) and spread to *Enterobacteriaceae* other than their species of origin.

The carbapenems

This preamble illustrates the amount of human effort that went into the development of this most useful group of antimicrobials and how at every turn the bacteria have been able to mount an appropriate response. The increasing resistance to penicillins and cephalosporins as a result of β-lactamases led to

some drug companies to screen soil actinomycetes for solutions. In the intervening years since the dawn of the age of antibiotics, drug discovery had become less likely to result from individual endeavour. In the 1970s, while looking for a β-lactamase inhibitor, researchers at Beecham Research Laboratories identified the olivanic acid complex from *Streptomyces olivaceus* (14). Almost simultaneously, thienamycin was being independently isolated by scientists at Merck from a strain of *Streptomyces cattleya* (15). These compounds differ from other β-lactams by having a carbon atom instead of sulphur in the fused ring structure. Merck scientists were eventually able to produce a N-formimidoyl derivative of thienamycin that overcame the inherent instability of the natural antibiotic. This resulted in imipenem, the first commercially available carbapenem which was launched in the late 1980s.

Imipenem delivered unprecedented activity against a broad spectrum of bacterial pathogens including Gram-positive bacteria and anaerobes. However it is against Gram-negative bacilli including *Pseudomonas aeruginosa* and *Acinetobacter* spp. that imipenem has found its main *raison d'etre*. This is because imipenem retains stability against extended-spectrum and AmpC β-lactamases. There remain a few issues with imipenem. Firstly, imipenem is hydroylzed by dehydropeptidase I which is naturally found in the kidney. Consequently imipenem has to be administered with a dehydropeptidase inhibitor called cilastatin. Imipenem is also perceived to be more prone to provoke seizures. This is thought to be related to the structure of the C-2 side chain which may interact with the γ-aminobutyric acid (GABAA) receptor

(16). Imipenem needs to be administered three or four times a day depending on the dose. It is not absorbed orally and therefore has to be given intravenously (17). Newer carbapenems are all attempts to overcome some of these problems.

Panipenem was introduced by Sankyo in 1993. However it still required a dehydropeptidase inhibitor, in this case betamipron (18). It does not offer any advantages over other presently available carbapenems and is virtually unknown outside the Japanese market.

Sumitomo Pharmaceuticals introduced the first commercial dehydropeptidase stable carbapenem when meropenem was launched in 1995. Meropenem is slightly more active against Gram-negative bacteria and slightly less active against Gram-positive bacteria *in-vitro* (19). It is also less prone to cause seizures compared with imipenem.

Merck launched ertapenem in 2002. This is excreted less quickly allowing for once-daily dosing. However, ertapenem has no clinically useful activity against *P. aeruginosa* and *Acinetobacter* spp. (20).

Of the remaining available carbapenems, biapenem resembles meropenem in its stability to dehydropeptidase and activity against Gram-negative bacteria

but is not widely used (21). Doripenem was launched in 2005 and also resembles meropenem in activity. *In vitro* studies show that doripenem may be less selective for resistant *P. aeruginosa* mutants compared with other carbapenems (22). Faropenem and ritipenem are penems that are similar in structure to carbapenems except that they have a sulphur in the β-lactam ring instead of carbon. They can be administered orally (23).

Because of their broad spectrum of antimicrobial activity and their stability to hydrolysis by most Gram-negative β-lactamases, the carbapenems have become the treatment of choice against multidrug-resistant Gram-negative bacilli, and as empirical therapy in critically ill patients with severe healthcare-associated infections.

Carbapenem resistance in Gram-negative bacilli

Carbapenem resistance may arise in Gram-negative bacilli via a number of different mechanisms. *Acinetobacter* spp. may develop reduced susceptibility to carbapenems because of alterations in the penicillin-binding protein targets (24). In *P. aeruginosa*, specific resistance to imipenem may result from impermeability due to diminished expression of the OprD porin (25). Reduced susceptibility to carbapenems in general may also arise in this species as a result of up-regulated efflux through the MexA-MexB-OprM pump (26). However, these mechanisms are intrinsic to each individual strain and the resistance is not transferable. Enzymes with specific hydrolytic activity (β-

lactamases) represent the most potent threat to the β-lactam antibiotics because they are able to confer much higher levels of resistance and the genes are potentially transferable. In reality, a combination of mechanisms may be responsible for the final antimicrobial resistance phenotype (27).

β-lactamase classification

There are currently several hundred different β-lactamases described. Variants of the original TEM and SHV alone number 205 and 168 respectively (http://www.lahey.org/Studies/ accessed on 29 December 2012). Two systems exist to classify β-lactamases. A functional scheme relies on the hydrolysis and inhibition profile of the enzyme (28), whereas a structural scheme is based on the molecular structure (29). As the number of β-lactamases increased in number and complexity, the structural classification by Ambler has largely superseded the latter on account of its logic and simplicity. This scheme divides the β-lactamases into 4 classes labelled A to D. There is some correlation with the functional classification. Classes A, C, and D have a serine at their active site. Class A β-lactamases may be penicillinases and/or cephalosporinases that are inhibited by clavulanic acid. Class D (OXA) β-lactamases are a very diverse group that are defined by their ability to hydrolyze oxacillin at a higher rate than penicillin, a property not seen in most class A and class C β-lactamases. They lack a specific inhibitor. Class C β-lactamases are cephalosporinases that are inhibited by cloxacillin. Class B β-lactamases are carbapenemases that are also known as metallo-β-lactamases

(MBL) because they require zinc ions at the active site and are therefore inhibited by metal-chelators.

Constitutive carbapenemases

Given that carbapenems are derived from natural products, it is not surprising that a few species of bacteria naturally produce carbapenemases in their wild state. Some are not clinically significant, like FEZ found in *Fluoribacter* (formerely *Legionella*) *gormani* (30). Others are Gram-negative bacilli that have low pathogenicity but may occasionally cause true infections that are difficult to treat because of intrinsic multidrug resistance. These include *Stenotrophomonas maltophilia* and *Elizabethkingae meningosepticum* that produce L1 (31), and GOB and BlaB (32) respectively. While problematic at the level of the individual patient, these constitutive carbapenemases present a limited threat at a global level. This is because the carbapenemase gene is on the chromosome and is not on a transferable element that may enable its spread to other potentially more pathogenic bacteria.

Acquired carbapenemases

The first plasmid-encoded carbapenemase was a class B enzyme called IMP-1, that was first described in *Serratia marcescens* in Japan in 1994 and subsequently found to have also spread among *P. aeruginosa* (33). The next family of plasmid-encoded class B enzymes were the VIM-type enzymes first described in *P. aeruginosa* in Italy in 1997 (34). Whereas IMP-1 and its

variants are found predominantly in Asia, VIM-type enzymes dominate in Europe particularly in Italy (35) and Greece (36). With 42 variants of IMP and 37 variants of VIM (http://www.lahey.org/Studies/ accessed on 29 December 2012) spread across 6 continents they have become the dominant class of carbapenemase in the last decade. A number of other plasmid-borne class B β-lactamases have since emerged but have remained relatively limited in their distribution. Recently a new class B enzyme has emerged from the Indian subcontinent. The gene encoding this new enzyme, $bla_{\text{NDM-1}}$, has spread at an unprecedented rate and the plasmids bearing the gene have demonstrated the ability to be easily transferred to other bacteria (37).

Following IMP and VIM, the next plasmid-associated carbapenemase to achieve international prominence was a class A enzyme. Class A carbapenemases were in fact some of the earliest carbapenemases to be discovered. SME-1 from *S. marcescens* isolated in London in 1982 (38) and IMI-1 from 2 *E. cloacae* isolated in a California hospital in 1984 (39) were both discovered before imipenem was introduced. The similar NMC-A was first described from an *E. cloacae* isolated in Paris in 1990 (40). Unusually, these enzymes hydrolyze carbapenems more strongly than oxyimino-cephalosporins. They were not found on transferable elements and remain rare. However in 1996, a carbapenem-resistant *Klebsiella pneumoniae* was isolated in North Carolina that produced a novel plasmid-associated class A carbapenemase (originally called KPC-1 but now revised to KPC-2) (41). The KPC family of carbapenemases presently number 13 variants (http://www.lahey.org/Studies/ accessed on 30 December 2012). Unlike

earlier, class A carbapenemases, KPC also strongly hydrolyzes oxyimino-cephalosporins. KPC producers have since spread along the eastern United States and have also been found overseas in Europe, Columbia, Israel and China largely as the result of spread of a particular clone designated ST258 by multilocus sequence typing (MLST) (42).

OXA carbapenemases were first found in the *Acinetobacter calcoaceticus-Acinetobacter baumannii* species complex (Acb) and most of them are still confined to that species complex. The gene for OXA-51-type enzymes ($bla_{\text{OXA-51-type}}$) is native to the chromosome of *A. baumannii* (43). On the other hand $bla_{\text{OXA-23, -24}}$ and $_{\text{-58-types}}$ are acquired and may be found on plasmids (44-46). Of these, $bla_{\text{OXA-23-type}}$ is largely responsible for the worldwide emergence of carbapenem-resistant *A. baumannii* and has been found in isolates dating back to 1985 (47). OXA-48 was first identified in *K. pneumonia*e in Turkey in 2003 (48). It remains the major cause of outbreaks of carbapenem-resistant *Enterobacteriaceae* in Turkey. OXA-48 producers have since spread to Europe and northern Africa (42). A new variant, OXA-181 has recently been found in India and, if the experience with NDM-1 is any precedent, seems likely to spread further abroad (49).

Taken together, the recent global spread of carbapenemase genes threatens the utility of this critical line of defence against multidrug-resistant bacilli and raises the spectre of a return to the age before antibiotics.

Mobilization of antimicrobial resistance genes in Gram-negative bacilli

There are many mechanisms by which genes may be acquired and disseminated between Gram-negative bacilli, though not all of them are known to be involved in the spread of carbapenemase genes. Transformation is the process by which bacteria acquire naked DNA from their surroundings. Transduction is the transfer of DNA between bacterial cells via a bacteriophage. While *Acinetobacter* spp. are naturally transformable and β-lactamase genes have been found on bacteriophages, both transformation and transduction are not normally thought to be significant mechanisms for the acquisition of resistance genes in Gram-negative bacilli (50, 51).

Insertion sequences (IS) are genetic elements that encode a transposase which is responsible for their excision and insertion into chromosomes and plasmids. They are able to transfer genetic material by a number of mechanisms. Two IS may flank an antimicrobial resistance gene to form a composite transposon as in the case of Tn*1999* and $bla_{\text{OXA-48}}$ (52). A single IS may also be able to mobilize adjacent DNA such as IS*Ecp1* associated with $bla_{\text{OXA-181}}$ (53).

Conjugation is a process to transfer DNA that requires cell-to-cell contact. Plasmids are double–stranded DNA, usually circular, that are capable of replication independent of the bacterial chromosome. Conjugative plasmids are the most common vectors for antimicrobial resistance genes between bacterial cells and can transfer the largest amounts of DNA. It has been

observed that resistance genes seem to be moving from chromosomes to plasmids more rapidly than ever before and that plasmids seem to be aggregating more resistance genes (54). Plasmids belonging to certain incompatibility groups are known to be more successful at spreading resistance genes and within these groups the structure of the plasmid is relatively conserved (55). However, exactly what makes these plasmids more successful at spreading resistance genes than others remains unknown.

Integrative and conjugative elements (ICEs) also mobilize genes by conjugation but unlike plasmids are not self-replicating and cannot exist independently of the chromosome (56). However, they have the ability to excise, initiate conjugation with a recipient cell and reintegrate into the new chromosome to be passed from one generation to the next. At the present time, no carbapenemase gene has been associated with ICEs.

The genes encoding IMP and VIM are often found on integrons. These are genetic units that are able to collect small mobile elements called gene cassettes via site-specific recombination. They are characterized by an *intI* gene that encodes a site-specific recombinase IntI and an *attI* recombination site recognized by the IntI integrase. The gene cassettes contain the antibiotic resistance gene and an *attC* recombination site that allows them to be recognized by IntI. Integrons are not mobile by themselves but may be associated with transposons and hence move onto plasmids (57).

Acquired carbapenem resistance in Gram-negative bacilli in Singapore

Acquired carbapenemase genes were discovered in Singapore very soon after they were first described. A carbapenem-resistant *K. pneumoniae* producing IMP-1 was isolated from a haematology patient with no apparent history of travel in 1996. This was the first time IMP-1 had been described outside Japan, and Singapore became only the third country (after Japan and Italy) to report a plasmid-borne carbapenemase gene (58).

Because $bla_{\text{IMP-1}}$ had been found in *P. aeruginosa* in Japan, a study was carried out to determine if this gene was present in this species in Singapore (59). Between December 1999 and February 2001, 36 isolates of carbapenemase-producing *P. aeruginosa* were collected. Pulsed-field gel electrophoresis (PFGE) showed that there were two clones. One clone had $bla_{\text{IMP-1}}$ that was identical in sequence to that first reported in Japan. The other clone had a variant with four silent mutations. A single isolate had $bla_{\text{IMP-7}}$. During the course of this study, four carbapenemase-producing fluorescent *Pseudomonas* spp. were isolated (60). One *Pseudomonas putida* had the original $bla_{\text{IMP-1}}$ sequence while a *Pseudomonas fluorescens* had the variant sequence. The remaining two *P. putida* isolates had identical PFGE patterns and produced a novel MBL, VIM-6, that differed from VIM-3 and VIM-2 by one and two amino acid residues respectively.

Acb were studied next because they are often carbapenem-resistant and isolated together with *P. aeruginosa* from the same patients. One hundred and fourteen isolates of imipenem-resistant Acb were collected over two 5-month periods (in 1996 and 2001) (61). The incidence of carbapenemase genes approximately doubled (3.7% to 7.2%) between the two study periods. PFGE showed a number of clones which were essentially different during the two study periods. There was a clear demarcation in the distribution of acquired carbapenemase genes. Carbapenem-resistant *A. baumannii* had $bla_{\text{OXA-23}}$ whereas carbapenem-resistant *Acinetobacter pittii* and *Acinetobacter nosocomialis* had $bla_{\text{IMP-4}}$ and $bla_{\text{OXA-58}}$ (62).

These early studies showed that clonal spread was occurring in these species. However the typing method employed did not allow comparison with clones in other countries. This was at a time when it was becoming apparent that spread of antimicrobial resistance worldwide was sometimes driven by specific international clones. In the meantime, new typing methods like MLST had been developed that allowed objective inter-laboratory comparison of strains and their evolutionary relationships (63).

The impetus for this study was the increasing problem of carbapenem-resistance among Gram-negative bacilli in Singapore. In a recent national survey, about 8% of *P. aeruginosa* clinical isolates in Singapore were resistant to carbapenems (64). The percentage of isolates of Acb that were resistant to carbapenems was even more alarming at about 65%. The percentage in cases

of bacteraemia was hardly better at 60% (65). This percentage of carbapenem resistance is one of the highest in the world. By comparison, carbapenem resistance in cases of *Acinetobacter* spp. bacteraemia in the United Kingdom in 2008 was around 15% (66). While carbapenem resistance among *Escherichia coli* and *K. pneumoniae* in Singapore was still too low to be expressed as a percentage, the absolute numbers appear to be on the increase (67, 68). Initially at least, most of the carbapenem resistance among *Enterobacteriaceae* was due to either pAmpC or CTX-M β-lactamases (69, 70). Between 1996 and 2010, only one other carbapenemase-producing *Enterobacteriaceae* was isolated. This was another IMP-1-producing *K. pneumoniae* isolated in 2004.

The aim of this thesis was to identify the reasons behind this increase in carbapenem resistance. The hypothesis was that the increase of carbapenem-resistance in Gram-negative bacilli in Singapore may be the result of the introduction of clones of carbapenemase-producers that have demonstrated the ability to spread globally.

Chapter 2: Acquired Carbapenemases in *P. aeruginosa*

Introduction

Carbapenem-resistant *P. aeruginosa* are an increasing problem worldwide. While many underlying mechanisms may account for carbapenem resistance in this species, the possession of MBL genes is of particular concern because these enzymes are able to hydrolyze all β-lactam antimicrobials with the exception of aztreonam. In addition, these genes may be mobilized and transferred between different species of bacteria. We conducted a study in 2008 to investigate if there were any changes in the epidemiology of *P. aeruginosa* isolates containing MBL genes in our hospital compared to an earlier survey carried out in 2000 (59).

Materials and methods

We studied *P. aeruginosa* isolated in the Singapore General Hospital from patients with suspected clinical infections in 2008 that were imipenem-resistant by CLSI criteria (71).

Modified Hodge test

Imipenem-resistant *P. aeruginosa* were screened for carbapenemase production by the modified Hodge test (72). A lawn of susceptible *E. coli* (ATCC 25922) was spread across a BBL™ Mueller Hinton II plate (BD,

Maryland, USA) using a cotton swab impregnated with a suspension of the organism adjusted to one-tenth the turbidity of a 0.5 McFarland standard. After brief drying, a 10 µg imipenem disk (BD) was placed in the centre of the plate and test and control organisms were heavily streaked from the edge of the disk to the edge of the agar on the plate. The plate was incubated in air overnight at 35ºC. The following day, any growth of *E. coli* along a streak into the zone of inhibition around the imipenem disk was interpreted as probable carbapenemase production by the organism in the streak. *K. pneumoniae* DB44384/96 (*bla*$_{\text{IMP-1}}$ positive) and *P. aeruginosa* ATCC 27853 were used as positive and negative controls respectively.

Imipenem-EDTA disk diffusion

Probable carbapenamease producers were tested for MBL production by the imipenem-EDTA disk diffusion test (73). A suspension of test isolate adjusted to match the turbidity of a 0.5 McFarland standard was inoculated onto a BBL$^{\text{TM}}$ Mueller Hinton II plate (BD) with a cotton swab. Two 10 µg imipenem disks (BD) were placed onto the agar and 750 µg of EDTA was applied to one of the disks. EDTA is a metal chelator and therefore inhibits MBLs that rely on zinc ions at their binding site for their activity. An increase in diameter of the zone of inhibition around the imipenem-EDTA disk of ≥7 mm compared to the imipenem-only disk indicated the likely presence of a MBL.

Multiplex PCR for MBL genes

Isolates that were likely to produce a MBL were tested for *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{SPM-1}$, *bla*$_{GIM-1}$ and *bla*$_{SIM-1}$ using multiplex PCR (74).

Table 1 Primers used in multiplex PCR for MBL genes.

Gene	Name	Primer (5'-3')
bla$_{IMP}$	Imp-F	GGAATAGAGTGGCTTAAYTCTC
	Imp-R	CCAAACYACTASGTTATCT
bla$_{VIM}$	Vim-F	GATGGTGTTTGGTCGCATA
	Vim-R	CGAATGCGCAGCACCAG
bla$_{GIM-1}$	Gim-F	TCGACACACCTTGGTCTGAA
	Gim-R	AACTTCCAACTTTGCCATGC
bla$_{SPM-1}$	Spm-F	AAAATCTGGGTACGCAAACG
	Spm-R	ACATTATCCGCTGGAACAGG
bla$_{SIM-1}$	Sim-F	TACAAGGATTCGGCATCG
	Sim-R	TAATGGCCTGTTCCCATGTG

DNA was extracted by emulsifying a 1 µl loopful of bacteria in 100 µl InstaGene^{TM} Matrix (Bio-Rad Laboratories, Hercules, CA, USA). The mixture was heated to 100ºC for 15 min in a heating block to lyse the cells, and then centrifuged at 12,000 rpm for 10 min to retrieve the supernatant.

The PCR reaction mixture was made up using the QIAGEN multiplex PCR Kit (QIAGEN GmbH, Hilden, Germany) and 2 µl of DNA extract in a final reaction volume of 25 µl.

Multiplex PCR was carried out in a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The cycling conditions were: initial denaturation at 95ºC for 15 min, followed by 36 cycles of denaturation at 94ºC for 30 s, annealing at 52ºC for 40 s, and elongation at 72ºC for 50 s, followed by a final elongation step at 72ºC for 5 min.

After the reaction was completed, the PCR products were mixed with 6X loading dye and loaded into a gel made from 2% SeaKem® LE agarose (Cambrex Bio Science Rockland Inc, Rockland, ME, USA) in 0.5X Tris-Borate-EDTA (TBE) buffer. A 100 bp DNA ladder (Promega Corporation, Madison, WI, USA) was used to size the products. Electrophoresis was carried out in a MINI-SUBCELL® GT (BIO-RAD, Hercules, CA, USA) with power provided by a POWER PAC 300 (BIO-RAD) at a constant voltage of 110V for 50 min.

The gel was stained in GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA) for 1 h before viewing in a Gel Doc™ 2000 (BIO-RAD).

Amplification and sequencing of MBL genes

The entire MBL gene was amplified and sequenced from DNA extracts that were positive by multiplex PCR using the primers described by Chu *et al* (75) and Yan *et al* (76).

Table 2 Primers used for amplification and sequencing of MBL genes.

Gene	Name	Primer (5'-3')
bla$_{IMP}$ amplification sequencing	IMP-13	ATCCAAGCAGCAAGCGCGTTA
	IMP-14	AGGCGTGCTGCTGCAACGACTTGT
bla$_{VIM-2}$ amplification	VIM-2ASF	AAAGTTATGCCGCACTCACC
	VIM-2ASR	TGCAACTTCATGTTATGCCG
bla$_{VIM-2}$ sequencing	VIM-2SF	TTGATGTCCTTCGGGCGGCT
	VIM-2SR	TCGACGGTGATGCGTACGTT

The PCR reaction mixture was made up using the QIAGEN *Taq* PCR Master Mix Kit (QIAGEN) and 2 µl of DNA extract in a final reaction volume of 25 µl. Amplification and sequencing were carried out in a GeneAmp® PCR System 9700 (Applied Biosystems). The cycling conditions were: initial denaturation at 94ºC for 3 min, followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min, and elongation at 72ºC for 2 min, followed by a final elongation step at 72ºC for 7 min.

The PCR products were cleaned up using the QIAquick PCR Purification Kit (Qiagen) to remove residual nucleotides, primers and *Taq* polymerase. 2.5 µl of purified PCR product was added to 1.5 µl of diluted primer (approximately 30 µM), 4 µl of ABI PRISM® BigDye Terminator (Applied Biosystems) and 2 µl of RNase-free water. The cycling conditions were: initial denaturation at 96ºC for 3 min, followed by 25 cycles of denaturation at 96ºC for 10 s, annealing at 50ºC for 5 s, and elongation at 60ºC for 4 min.

Absolute ethanol (25 µl) and 1 µl 3M sodium acetate were added to the cycle sequencing products which were left at room temperature for 30 min to precipitate. After centrifugation at 12,000 rpm for 20 min, the supernatant was discarded. The pellet was washed with 125 µl of 70% ethanol and centrifuged again at 12,000 rpm for 10 min. After removing the supernatant, the pellet was re-suspended in 12 µl Hi-Di™ Formamide (Applied Biosystems). The prepared cycle sequencing products were denatured at 95ºC for 2 min using a GeneAmp® PCR System 9700 (Applied Biosystems) before loading onto an ABI® PRISM 3100 Genetic analyser (Applied Biosystems).

The obtained sequences were edited and assembled using ChromasPro (Technelysium, South Brisbane, QLD, Australia) and compared with sequences held on the GenBank database using BLAST.

PFGE

The clonal relationship between isolates with MBL genes was determined by PFGE of chromosomal DNA restricted with *Spe*I (59).

Isolates of interest were cultured on tryptic soy agar containing 5% sheep blood (BD). After overnight growth in air at 35ºC, 1-5 colonies were picked and inoculated into 6 ml of tryptic soy broth and incubated overnight at 35ºC.

Cells were harvested by centrifuging the broth culture at 2,800 rpm for 20 min. the supernatant was discarded and the pellet re-suspended in 1 ml of PIV buffer (Appendix 1). The suspension was transfered to a microcentrifuge tube, and washed twice by centrifuging at 13,000 rpm for 2 min, discarding the supernatant, and re-suspending the pellet in 1 ml of PIV buffer. After the final wash, the pellet was re-suspended in 200 µl of PIV buffer by vortexing. The optical density of the suspension was measured at a wavelength of 620 nm using a GENESYS™ 10UV spectrophotometer (Thermo Spectronic, Rochester, NY, USA) and the final cell concentration adjusted to an optical density (O.D.) of 5.0 by adding PIV buffer.

Plugs were made by adding 100 µl pre-melted 2% SeaPlaque® GTG® agarose (FMC Bioproducts, Rockland, ME, USA) held at 50ºC to 100 µl of the $O.D._{620}$ 5.0 cell suspension in a microcentrifuge tube and mixing gently. The

mixture was dispensed into plug molds (BIO-RAD) and left to cool at 4ºC for 30 min.

The set gel plugs were carefully pushed out of the molds into individual wells of a six-well tissue culture plate containing 2 ml of EC lysis buffer (Appendix 1) per well.

The cells in the plugs were lysed by adding 10 µl of lysozyme (20 mg/ml) and 10 µl of RNase (10 mg/ml) to each well and incubating at 35ºC for at least 3 h. The EC lysis solution was then aspirated with a Pasteur pipette and discarded.

Contaminating proteins in the plugs was digested by adding 2 ml of ES buffer (Appendix 1) to each well followed by 100 µl of Proteinase K (20 mg/ml) and incubating overnight at 50ºC. The ES buffer was then aspirated and the plugs washed at least 5 times with 5 ml of TE buffer.

Restriction digestion of the DNA in the plugs was performed in 96-well tissue culture plates. Each well contained 4 µl of 10X restriction enzyme buffer, 36 µl of DNase/RNase-free water, 1 µl of restriction enzyme and 1 µl of bovine serum albumin. The plugs were then incubated at 35ºC for the recommended time.

The digested plugs were placed in a 1% SeaKem® GTG® (Cambrex Bio Science Rockland Inc) agarose gel together with a Lambda ladder (BIO-RAD) and run in 0.5X TBE in a CHEF MAPPER (BIO-RAD). The initial switch time was 5.3 s with a final switch time of 34.9 s using a linear ramp at 6 V/cm run for 19.5 h. After completion, the gel was stained in GelRed™ Nucleic Acid Gel Stain (Biotium) for 1 h before viewing in a Gel Doc™ 2000 (BIO-RAD).

The PFGE band patterns were analyzed in Bionumerics (Applied Maths NV, Sint-Martens-Latem, Belgium) and all strains with more than 85% similarity were considered to belong to the same clone.

MLST

All MBL-producing isolates were further subjected to MLST (77). This typing method is based on sequencing the following seven house-keeping genes in the *P. aeruginosa* chromosome; *acsA* (acetyl coenzyme A synthetase), *aroE* (ahikimate dehydrogenase), *guaA*(GMP synthase), *mutL* (DNA mismatch repair protein), *nuoD* (NADH dehydrogenase I chain C, D), *ppsA* (phosphoenolpyruvate synthase), and *trpE* (anthralite synthetase component I). MLST is able to characterize bacterial types in an unambiguous fashion and establish evolutionary relationships between strains better than band based methods like PFGE.

The PCR reaction mixture was made up using the QIAGEN *Taq* PCR Master Mix Kit as described above. The cycling conditions for amplification were: initial denaturation at 96ºC for 1 min, followed by 30 cycles of denaturation at 96ºC for 1 min, annealing at 55ºC for 1 min, and elongation at 72ºC for 1 min, followed by a final elongation step at 72ºC for 10 min.

Table 3 Primers used for amplification of house-keeping genes for *P. aeruginosa* MLST.

Gene	Name	Primer (5'-3')
acsA	acsA-F	ACCTGGTGTACGCCTCGCTGAC
	acsA-R	GACATAGATGCCCTGCCCCTTGAT
aroE	aroE-F	TGGGGCTATGACTGGAAACC
	aroE-R	TAACCCGGTTTTGTGATTCCTACA
guaA	guaA-F	CGGCCTCGACGTGTGGATGA
	guaA-R	GAACGCCTGGCTGGTCTTGTGGTA
mutL	mutL-F	CCAGATCGCCGCCGGTGAGGTG
	mutL-R	CAGGGTGCCATAGAGGAAGTC
nuoD	nuoD-F	ACCGCCACCCGTACTG
	nuoD-R	TCTCGCCCATCTTGACCA
ppsA	ppsA-F	GGT CGC TCG GTC AAG GTA GTG G
	ppsA-R	GGGTTCTCTTCTTCCGGCTCGTAG
trpE	trpE-F	GCGGCCCAGGGTCGTGAG
	trpE-R	CCCGGCGCTTGTTGATGGTT

The amplified PCR products were cleaned up as described previously and sent to a commercial company for sequencing (AITbiotech, Singapore).

Table 4 Primers used for sequencing of house-keeping genes for *P. aeruginosa* MLST.

Gene	Name	Primer (5'-3')
acsA	acsA-F	GCCACACCTACATCGTCTAT
	acsA-R	AGGTTGCCGAGGTTGTCCAC
aroE	aroE-F	ATGTCACCGTGCCGTTCAAG
	aroE-R	TGAAGGCAGTCGGTTCCTTG
guaA	guaA-F	AGGTCGGTTCCTCCAAGGTC
	guaA-R	GACGTTGTGGTGCGACTTGA
mutL	mutL-F	AGAAGACCGAGTTCGACCAT
	mutL-R	GGTGCCATAGAGGAAGTCAT
nuoD	nuoD-F	ACGGCGAGAACGAGGACTAC
	nuoD-R	TGGCGGTCGGTGAAGGTGAA
ppsA	ppsA-F	GGTGACGACGGCAAGCTGTA
	ppsA-R	GTATCGCCTTCGGCACAGGA
trpE	trpE-F	TTCAACTTCGGCGACTTCCA
	trpE-R	GGTGTCCATGTTGCCGTTCC

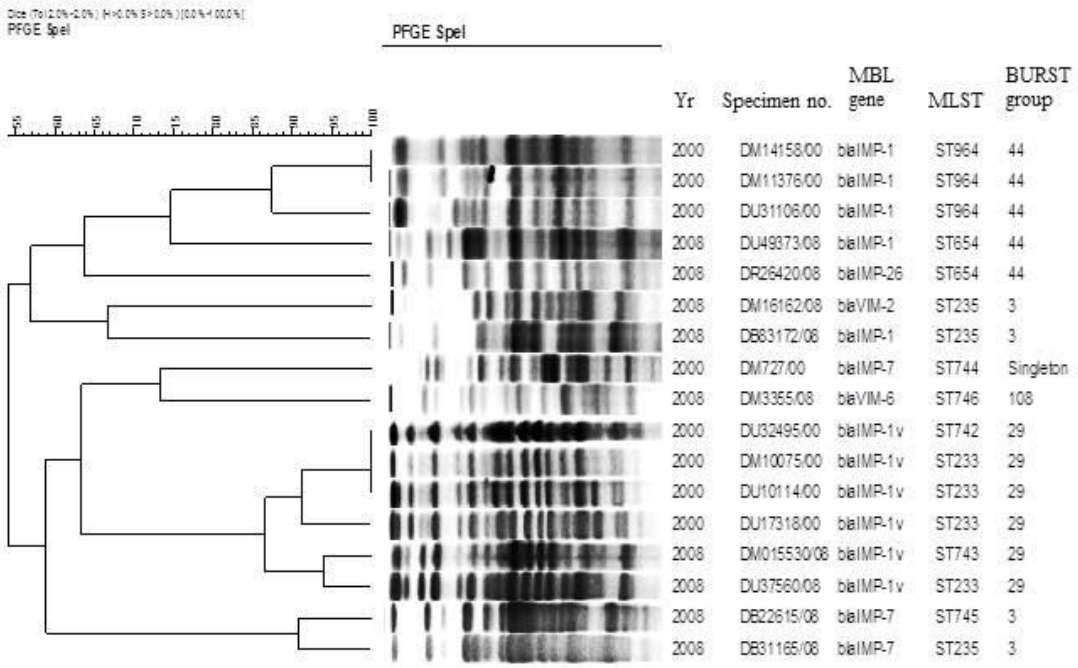
MLST profiles were obtained by submitting the sequences to the *P. aeruginosa* MLST Database (http://pubmlst.org/paeruginosa/). MLST profiles were submitted to eBURST V3 (http://eburst.mlst.net/) on 19 January 2013. Isolates sharing six out of seven alleles were assigned to the same BURST

group and were considered to belong to the same clonal complex descended from a common founder genotype. Representative MBL-producing *P. aeruginosa* from the 2000 survey were also subjected to PFGE and MLST.

Results

One hundred and twenty-three isolates of 2,552 non-duplicate *P. aeruginosa* isolated in 2008 were imipenem-resistant. Nine isolates were positive for carbapenemase by the modified Hodge test, MBL production by imipenem-EDTA disk diffusion, and multiplex PCR for MBL genes. Seven representatives from the two major clones in 2000 that carried $bla_{\text{IMP-1}}$ or its variant $bla_{\text{IMP-1v}}$, and the solitary isolate with $bla_{\text{IMP-7}}$ were also re-investigated for comparison. The PFGE, MBL gene sequence and MLST results are summarized in Figure 1. The eBURST anaysis showed that ST233 is the founder of a small clonal complex including ST742 and ST743 (CC233 or BURST Group 29). ST235 is a founder of a large clonal complex (CC235 or BURST Group 3) that includes ST745 and ST654 is a founder of a small complex including ST964 (CC654 or BURST Group 44).

Figure 1 Dendrogram of PFGE patterns of *P. aeruginosa* isolates with MBL genes. The source of the isolate is indicated by the 2 letter prefix of the specimen number: DM; miscellaneous (including wounds), DU; urine, DR; respiratory; DB; blood. Reprinted with permission. Copyright © American Society for Microbiology, Journal of Clinical Microbiology, Vol. 48, 2010, p. 2563–2564, doi:10.1128/JCM.01905-09.



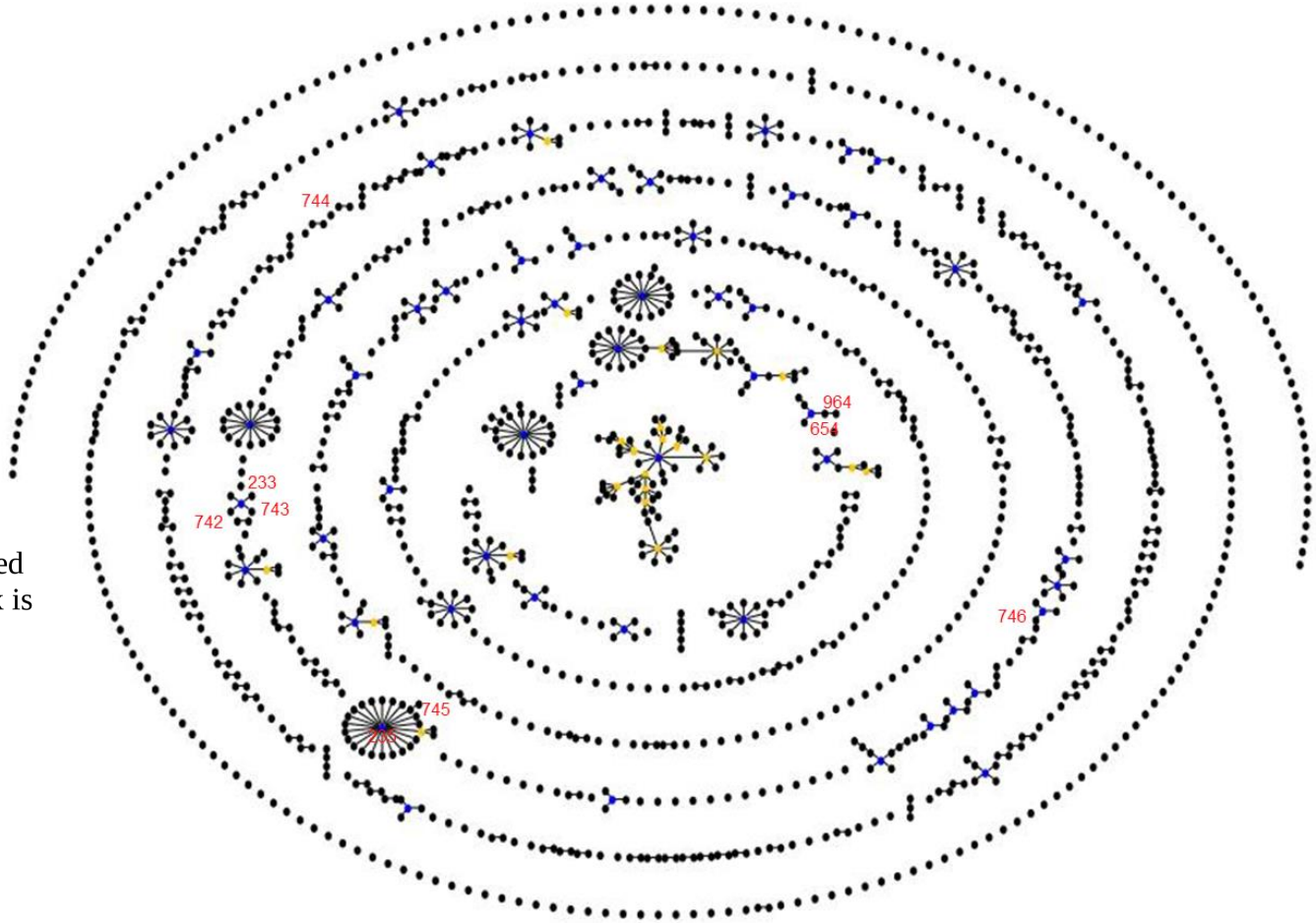


Figure 2 ‘Population Snapshot’ showing the clusters of linked and unlinked STs in the whole *P. aeruginosa* MLST database (19 January 2013). ST labels have been removed except for those of isolates from this study (in red). The predicted primary founder of a clonal complex is coloured blue and the subgroup founder is coloured yellow.

Discussion

In our previous study, 21 of 2,094 (1.0%) of all non-duplicate *P. aeruginosa* isolates in our hospital had MBL genes (59). With the exception of 1 isolate with $bla_{\text{IMP-7}}$, all other isolates had $bla_{\text{IMP-1}}$ and belonged to 1 of 2 PFGE clones. Isolates belonging to clone A had sequences identical to that of the original $bla_{\text{IMP-1}}$ first reported in Japan. Four representatives of clone A isolated from our hospital in 2000 had ST964 by MLST. Isolates belonging to clone B isolated in 2000 had sequences for variant $bla_{\text{IMP-1}}$ ($bla_{\text{IMP-1v}}$) with 4 silent mutations. Three representatives of this clone from 2000 had ST233 and one had ST742 by MLST. All four representatives of clone B belong to the same BURST group which was different from that of clone A.

In contrast, in the 2008 survey, 9 of 2,552 (0.35%) non-duplicate *P. aeruginosa* had MBL genes. Unlike the earlier study, there were no large clonal outbreaks. Two isolates with $bla_{\text{IMP-1v}}$ had similar PFGE patterns and belonged to the same BURST group as representative isolates from clone B in 2000.

Two isolates from 2008 with $bla_{\text{IMP-7}}$ had similar PFGE patterns and shared the same BURST group. The rest of the isolates from 2008 had distinct PFGE patterns.

There was a greater diversity of MBL genes compared to the 2000 survey. In particular, this is the first time that $bla_{\text{VIM-2}}$, and $bla_{\text{VIM-6}}$, have been found in *P. aeruginosa* in Singapore. $bla_{\text{IMP-26}}$ is a novel MBL gene that differs from

bla$_{IMP-4}$ at position 145 (G to T). The translated amino acid sequence differs from IMP-4 at residue 49 (Phenylalanine for Valine). This sequence has been previously deposited in the GenBank database as IMP-4 in an *A. calcoaceticus* from Malaysia (accession number ABC24668.1).

Three of the isolates in this study (separately containing *bla*$_{VIM-2}$, *bla*$_{IMP-1}$, and *bla*$_{IMP-7}$) belonged to ST235. This sequence type has been described in a VIM-producing *P. aeruginosa* isolate in Belgrade and is the founder of an international clonal complex of isolates bearing MBL genes found in several countries in Europe (78). Recently, an increasing prevalence of IMP-1-producing *P. aeruginosa* has been found in Hiroshima, Japan. This was due entirely to the clonal expansion of only two lineages, ST235 (BURST group 3) and ST357 (BURST group 108) (79). This is similar to the situation that existed in Singapore in 2000 where only two lineages (BURST groups 29 and 44) accounted for the majority of MBL-producing *P. aeruginosa* (59).

It is noteworthy that the original fear that a clone of MBL-producing *P. aeruginosa* would become established in Singapore has not been realized. The BURST group 29 and 44 lineages from 2000 are represented by only 1-2 isolates in 2008. The 2 *P. aeruginosa* with *bla*$_{IMP-7}$ in 2008 are unrelated to the solitary isolate with *bla*$_{IMP-7}$ in 2000. It has been suggested that *P. aeruginosa* displays an epidemic population structure with a limited number of clones emerging from a large number of unrelated genotypes (80). Although we did not correlate our study with hospital infection control measures, the Japanese data and our own seems to suggest that controlling the prevalence of MBL-

producing *P. aeruginosa* may be achieved by preventing the transmission of specific epidemic clones.

While it is reassuring to note that the prevalence of MBLs in carbapenem-resistant *P. aeruginosa* has not increased, the increased diversity of MBL genes represents a new cause for concern. Although clones of MBL-producing *P. aeruginosa* have not become established, it seems likely, given the variation of MBL genes and MLST types in this study, that MBL-producing *P. aeruginosa* continue to be introduced to our hospital from diverse sources.

Chapter 3 Acquired Carbapenemases in Acb

Introduction

The Acb comprises *A. calcoaceticus*, *A. baumannii*, *A. pittii* and *A. nosocomialis*. *A. calcoaceticus* is rarely found in clinical specimens whereas *A. baumannii* is a well-established pathogen. The significance of *A. pittii* and *A. nosocomialis* is largely unknown because Acb are not routinely identified to species level in the clinical laboratory.

The increasing prevalence of multidrug-resistant Acb, in particular carbapenem-resistant *A. baumannii* in several Asia countries is alarming. We also noted this trend in one preliminary study in Singapore (62). The current study was carried out to determine the prevalence, distribution of specimen sources, and antimicrobial susceptibility of Acb in Singapore.

Materials and methods

Bacterial isolates

A total of 193 serial unselected non-replicate Acb clinical isolates were collected from patients with suspected clinical infection in 6 hospitals [Alexandra Hospital (Hospital A), KK Women's and Children's Hospital (K), National University Hospital (N), Changi General Hospital (C), Singapore

General Hospital (S), and Tan Tock Seng Hospital (T)] in Singapore over a 1-month period in 2006.

Identification of *A. baumannii* by one tube multiplex PCR

Identification of *A. baumannii* was carried out by a one tube multiplex PCR targeting an internal 208 bp fragment from the intergenic spacer (ITS) region of *A. baumannii* and a highly conserved 425 bp region of the *recA* gene of *Acinetobacter* spp. (81). Clinical isolates of *A. baumannii* and *Acinetobacter lwoffi* were used as controls.

Table 5 Primers used in one tube multiplex PCR for identification of *A. baumannii.*

Target	Name	Primer (5'-3')
ITS	P-Ab-ITSF	CATTATCACGGTAATTAGTG
	P-Ab-ITSB	AGAGCACTGTGCACTTAAG
recA	P-rA1	CCTGAATCTTCTGGTAAAAC
	P-rA2	GTTTCTGGGCTGCCAAACATTAC

Multiplex PCR was carried as described in Chapter 2. The cycling conditions were: initial denaturation at 95ºC for 15 min, followed by 30 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s, and elongation at 72ºC for 30 s, followed by a final elongation step at 72ºC for 7 min.

Identification of Acb other than *A. baumanii* by ITS sequencing

ITS sequencing was used for identification of other members of the Acb (82). The primers 1512F 5'-GTCGTAACAAGGTAGCCGTA-3' and 6R 5'-GGGTTYCCCCRTTCRGAAAT-3' were used to amplify a DNA fragment spanning from the 16S rRNA gene region to the small fragment of the 23S rRNA gene region. . The cycling conditions were: initial denaturation at 94ºC for 2 min, followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 62ºC for 1 min, and elongation at 72ºC for 1 min, followed by a final elongation step at 72ºC for 7 min. The PCR products were cleaned up and sequenced as described in Chapter 2 using the same primers used for amplification. The obtained sequences were trimmed at the 5' and 3' ends to yield the ITS sequence starting with ACGAAAGATT and ending with GGGGTTGTAT before querying the GenBank database to identify the species.

Confirmation of Acb species by amplified fragment length polymorphism (AFLP™) analysis

Selective amplification of restriction fragments using AFLP™ analysis was also used for confirmatory identification where necessary (83). The methodology of AFLP™ is given below.

Antimicrobial susceptibility testing

Minimal inhibitory concentrations (MICs) of sulbactam-ampicillin (SAM), piperacillin-tazobactam (TZP), ceftazidime (CAZ), cefepime (FEP), imipenem

(IPM), meropenem (MEM), amikacin (AMK), gentamicin (GEN), ciprofloxacin (CIP), polymixin B (POL), and tigecycline (TGC) were carried out by microbroth dilution using custom Sensititre plates (TREK Diagnostic Systems, LTD, East Grinstead, United Kingdom). *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used for quality control of plates upon delivery and once a week during testing. Antimicrobial susceptibilities were interpreted in accordance with the guidelines of the Clinical Laboratory Standards Institute (84), except for tigecycline where the manufacturer's breakpoints for *Enterobacteriaceae* were used.

PCR for OXA, MBL genes, and insertion sequences

We sought $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24-like}}$, $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-58-like}}$, and $bla_{\text{OXA-143}}$ carbapenemase genes by multiplex PCR (85, 86). The cycling conditions were: initial denaturation at 95ºC for 15 min, followed by 30 cycles of denaturation at 94ºC for 25 s, annealing at 52ºC for 40 s, and elongation at 72ºC for 50 s, followed by a final elongation step at 72ºC for 6 min.

Table 6 Primers used in multiplex PCR for OXA genes.

Gene	Name	Primer (5'-3')
$bla_{\text{OXA-51-like}}$	OXA-51-F	TAATGCTTTGATCGGCCTTG
	OXA-51-R	TGGATTGCACTTCATCTTGG
$bla_{\text{OXA-23-like}}$	OXA-23-F	GATCGGATTGGAGAACCAGA
	OXA-23-R	ATTTCTGACCGCATTTCCAT
$bla_{\text{OXA-24-}}$	OXA-24-F	GGTTAGTTGGCCCCCTTAAA

like	OXA-24-R	AGTTGAGCGAAAAGGGGATT
bla$_{\text{OXA-58-like}}$	OXA-58-F	AAGTATTGGGGCTTGTGCTG
	OXA-58-R	CCCCTCTGCGCTCTACATAC
bla$_{\text{OXA-143}}$	OXA-143-F	TGGCACTTTCAGCAGTTCCT
	OXA-143-R	TAATCTTGAGGGGGCCAACC

The presence of insertion sequences preceding the *bla*$_{\text{OXA}}$ genes in carbapenem-resistant (meropenem or imipenem MIC ≥ 8 mg/L) isolates was determined using the forward primers ISAba1B, ISAba2A, ISAba3C, and ISAba4B in combination with reverse primers for *bla*$_{\text{OXA}}$ genes (87, 88).

Table 7 Forward primers of insertion sequences.

Gene	Name	Primer (5'-3')
IS*Aba1*	ISAba1B	CATGTAAACCAATGCTCACC
IS*Aba2*	ISAba2A	AATCCGAGATAGAGCGGTTC
IS*Aba3*	ISAba3C	AGCAATATCTCGTATACCGC
IS*Aba4*	ISAba4B	ACTCTCATATTTTTTCTTGG

MBL genes were sought using the multiplex method previously described in Chapter 2 (74).

Random amplified polymorphic DNA (RAPD) analysis

Ninety-three isolates with an imipenem MIC ≥ 8 μg/ml were subjected to RAPD analysis (89). For RAPD using the M13 primer (5'-GAGGGTGGCGGTTCT-3'), the cycling conditions were: initial denaturation at 95ºC for 15 min, followed by 35 cycles of denaturation at 94ºC for 20 s,

annealing at 50ºC for 1 min, and elongation at 72ºC for 20 s, followed by a final elongation step at 72ºC for 5 min. For RAPD using the DAF4 primer (5'-CGGCAGCGCC-3'), the cycling conditions were 95°C for 15 minutes, followed by 45 cycles of denaturation at 94°C for 40 seconds, annealing at 45°C for 40 s, and elongation at 72°C for 40 s, followed by a final extension at 72°C for 5 minutes. The PCR products were run in 2% agarose gels in 0.5X TBE at 90V. The stained gel images were uploaded onto a computer and the band patterns analyzed in Bionumerics (Applied Maths NV).

AFLP™

AFLP™ was used to type ten isolates of carbapenem-resistant *A. baumannii* from the present study, and seven archived isolates from Hospital S. The latter included 5 carbapenem-resistant *A. baumannii* characterized from a previous study (61), a carbapenem-susceptible *A. baumannii* (DU53770), and the oldest carbapenem-resistant *A. baumannii* in the archive (DB24815 isolated in 1993 from a patient on the Burns unit).

DNA isolation was performed according to the method by Boom *et al* (90). A fresh bacterial culture was prepared on Mueller Hinton agar and incubated overnight at 30ºC. A grain-sized amount of culture was suspended in 100 µl of TE buffer in an Eppendorf tube. To this was added 1 ml of Lysis Buffer (Appendix 2) and the tube tilted until the fluid cleared. This was then mixed for 1 min with 40 µl of Celite (Janssen Chemica, Beerse, Belgium). The tubes were centrifuged for 20 s at 14,000 rpm. The supernatant was discarded

carefully using a vessel containing 5 N NaOH to avoid the formation of cyanide gas. The pellet was washed 2X with 900 µl Wash Buffer (Appendix 2), remembering to collect the supernatant in 5 N NaOH, 2X with 900 µl 70% non-denatured ethanol, and 1X with 900 µl acetone. The pellet was carefully homogenized at each step by stirring with the pipette tip. Each centrifugation step was performed at 14,000 rpm for 20 s. After the last step, as much of the fluid was discarded as possible and the pellet dried for 10 min in a heating block at 56ºC until the acetone smell was no longer present. The pellet was rehydrated and homogenized thoroughly with 100 µl of TE buffer and the tubes placed in a shaking water bath at 56ºC. After this, the tubes were centrifuged for 5 min at 14,000 rpm and the supernatant transferred to a clean Eppendorf tube. This was incubated at 37ºC for 15 min with 25 µl RNAse (0.25 mg/ml). The DNA concentration was measured and diluted down to 10 ng/µl with milli-Q water (EMD Millipore, Billerica, MA, USA).

AFLP™ was performed as described by Nemec *et al* (91) using adapters described by Vos *et al* (92). *Acinetobacter* 15TU LUH 1091 was used as a control.

*Eco*RI adapter

5’-CTCGTAGACTGCGTACC
CATCTGACGCATGGTTAA-5’

*Mse*I adapter

5’-GACGATGAGTCCTGAG
TACTCAGGACTCAT-5’

Restriction digestion of 10-50 ng template DNA with 1U *Eco*RI (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) and 1U *Mse*I (New England Biolabs) was performed simultaneously with adapter ligation at 37ºC for 3 h in a 10 µl volume containing 4U T4 ligase (Amersham Pharmacia Biotech), 1X T4 DNA ligase buffer, 500 ng BSA, 50 mM NaCl, 2 pmol *Eco*RI adapters and 20 pmol *Mse*I adapters. After incubation, the mixture was diluted to a final volume of 200 µl with 10 mM Tris-HCl, 0.1 mM EDTA (pH 8.0).

PCR was performed in a final reaction volume of 10 µl containing 5 µl of the digestion/ligation mixture, 20 ng FAM-labelled *Eco*RI+A primer (FAM-GACTGCGTACCAATT Ca-3' ; where a is a selective A base), 60 ng *Mse*I+C primer (5'-GATGAGTCCTGAGTAAc-3' ; where c is a selective C base), 1X *Taq* polymerase buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP and 1 U Goldstar *Taq* DNA polymerase (Eurogentec, Maastricht, The Netherlands). The cycling conditions were 72°C for 2 min and 94°C for 2 min; 13 cycles of 94°C for 30 s, 65°C for 30 s with the annealing temperature lowered by 0.7°C for each subsequent cycle, 72°C for 60 s; 23 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 60 s; followed by a final elongation step of 72°C for 10 min.

The PCR products were mixed with 3 µl formamide containing 0.5% dextran blue, heated at 95°C for 5 min and cooled on ice. Samples of 3 µl were run in POP-4® Polymer (Applied Biosystems) and the amplicons were separated with an ALF express system (Amersham Pharmacia Biotech). The profiles with fragments of 50 to 500 bp were investigated by cluster analysis using Bionumerics 5.1 software (Applied Maths) with Pearson's coefficient for

similarity and UPGMA for grouping. Isolates were compared to each other and to a library of >2000 reference strains of all *Acinetobacter* spp. including taxonomically and epidemiologically defined strains. Isolates were identified to the same species, clone or strain if grouping at ≥50%, ≥ 80% or ≥90%, respectively.

MLST

Isolates representing significant clones were subjected to MLST using the Institut Pasteur scheme (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Abaumannii.html). This is based on sequencing the following seven house-keeping genes in the *A. baumannii* chromosome; *cpn60* (60-kDa chaperonin), *fusA* (elongation factor EF-G), *gltA* (citrate synthase), *pyrG* (CTP synthase), *recA* (homologous recombination factor), *rplB* (50S ribosomal protein L2), and *rpoB* (RNA polymerase subumit B).

Table 8 Primers used for amplification of house-keeping genes for *A. baumannii* MLST.

Gene	Name	Primer (5'-3')
cpn60	cpn60:F:cpn60F	ACTGTACTTGCTCAAGC
	cpn60:F:cpn60R	TTCAGCGATGATAAGAAGTGG
fusA	fusA:F:fusA7	ATCGGTATTTCTGCKCACATYGAT
	fusA:R:fusA8	CCAACATACKYTGWACACCTTTGTT
gltA	gltA:F:gltAF	AATTTACAGTGGCACATTAGGTCCC

	gltA:R:gltAR	GCAGAGATACCAGCAGAGATACACG
pyrG	pyrG:F:pyrG7	GGTGTTGTTTCATCACTAGGWAAAGG
	pyrG:R:pyrG8	ATAAATGGTAAAGAYTCGATRTCACCMA
recA	recA:F:RA1	CCTGAATCTTCYGGTAAAAC
	recA:R:RA2	GTTTCTGGGCTGCCAAACATTAC
rplB	rplB:F:rplB7	GTAGAGCGTATTGAATACGATCCTAACC
	rplB:R:rplB8	CACCACCACCRT GYGGGTGATC
rpoB	rpoB:F:Vic4	GGCGAAATGGC(AGT)GA(AG)AACCA
	rpoB:R:Vic6	GA(AG)TC(CT)TCGAAGTTGTAACC

The cycling conditions were: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 s, and elongation at 72°C for 30 s, followed by a final extension at 72°C for 5 min. Sequencing was performed in both directions with the same primers used for amplification. MLST profiles were submitted to eBURST V3 (http://eburst.mlst.net/) on 19 January 2013. Isolates sharing six out of seven alleles were assigned to the same BURST group and were considered to belong to the same clonal complex descended from a common founder genotype.

Results

Identification of Acb by 1 tube multiplex PCR, ITS sequencing and AFLP™.

Of the 193 isolates, 152 were identified to *A. baumannii* (78.7%) by multiplex PCR, and 18 to *A. pittii* (9.3%), and 23 to *A. nosocomialis* (11.9%) by ITS sequencing. Both AFLP™ and ITS sequencing were in agreement for the non-*baumannii* strains. The distribution of isolates according to the type of specimen is shown in Table 9. It is noteworthy that most (63.2% *A. baumannii*, 55.6% *A. pittii*, 60.9% *A. nosocomialis*) were recovered from respiratory and wound specimens and the respective proportions from blood for these species were 7.2%, 16.7% and 26.1%. *A. baumannii* and *A. pittii* were found in similar frequency from urine specimens (25.7% and 27.8%, respectively).

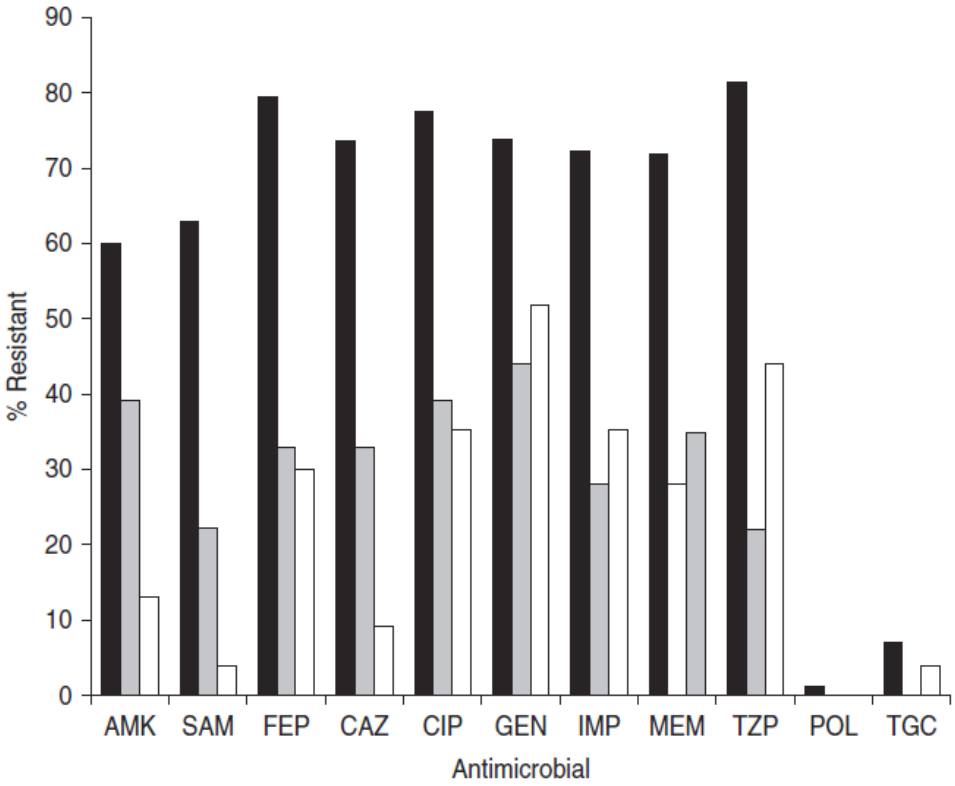
Table 9 Distribution of Acb by specimen of 193 clinical isolates from 6 hospitals in Singapore in 2006.

Specimen type	*A. baumannii*	*A. pittii*	*A. nosocomialis*
Respiratory tract	50 (32.9%)	5 (27.8%)	8 (34.8%)
Soft tissue	46 (30.3%)	5 (27.8%)	6 (26.1%)
Urinary tract	39 (25.7%)	5 (27.8%)	2 (8.7%)
Blood	11 (7.2%)	3 (16.7%)	6 (26.1%)
Line	5 (3.3%)	0	1 (4.3%)
Fluid	1 (0.7%)	0	0
Total	152	18	23

Antimicrobial susceptibility testing

The antimicrobial resistance profiles of the different species are shown in Figure 3. A high proportion of *A. baumannii* (110 isolates, 72.4%), but also 5 (27.8% of) *A. pittii* and 8 (34.8% of) *A. nosocomialis* were resistant to carbapenems. Overall, 150 isolates (77.7%) were multidrug-resistant, defined as resistant to 3 or more antimicrobial agents. This comprised 127 of *A. baumannii* isolates (83.6%), 11 of *A. pittii* isolates (61.1%), and 12 of *A. nosocomialis* isolates (52.2%).

Figure 3 Antimicrobial resistance profile of *A. baumannii* (black), *A. pittii* (grey), and *A. nosocomialis* (white). Reprinted with permission. Copyright © Cambridge University Press, Epidemiology and Infection, Vol. 140, 2010, p. 535–538, doi:10.1017/S0950268811001129.



PCR for OXA, MBL genes, and insertion sequences

One hundred and sixteen isolates (108 *A. baumannii*, 8 *A. nosocomialis*) were positive for $bla_{\text{OXA-23-like}}$. Of the isolates that were resistant to imipenem, IS*Aba1* was located upstream of this OXA-gene (IS*Aba1*-$bla_{\text{OXA-23-like}}$) in 70 *A. baumannii* and 7 *A. nosocomialis*. Only 2 imipenem-susceptible *A. baumannii* had $bla_{\text{OXA-23-like}}$. In both cases, there was no IS element upstream of the $bla_{\text{OXA-23-like}}$ gene. All *A. baumannii*, and 1 *A. nosocomialis* were positive for the $bla_{\text{OXA-51-like}}$ gene. Of the imipenem-resistant *A. baumannii*, IS*Aba1* was upstream of the OXA-51-like gene in only 12 isolates (IS*Aba1*-$bla_{\text{OXA-51-like}}$). In only 3 of these was IS*Aba1*-$bla_{\text{OXA-51-like}}$ likely to be the major contributor to imipenem resistance as the remainder also possessed IS*Aba1*-$bla_{\text{OXA-23-like}}$ concurrently.

Thirteen isolates were positive for $bla_{\text{OXA-58-like}}$ (1 *A. baumannii*, 4 *A. nosocomialis*, and 8 *A. pittii*). Among the imipenem-resistant isolates, this gene was preceded by IS*Aba3* (IS*Aba3*-$bla_{\text{OXA-58-like}}$) in 1 *A. nosocomialis* and 3 *A. pittii* isolates. Only 1 imipenem-resistant *A. baumannii* isolate (positive for $bla_{\text{OXA-51-like}}$, and $bla_{\text{OXA-23-like}}$, both lacking IS*Aba1* upstream) and one imipenem-resistant *A. pittii* (positive for *ISAba3*-$bla_{\text{OXA-58-like}}$) had $bla_{\text{IMP-like}}$. None of the isolates tested were positive with primers for $bla_{\text{OXA-24-like}}$, $bla_{\text{OXA-143}}$, IS*Aba2*, or IS*Aba4*.

RAPD analysis

Two isolates failed to produce patterns with the M13 primer. The RAPD PCR patterns using DAF4 and M13 primers were difficult to interpret and

discrepant. Because the DAF4 RAPD PCR patterns were complete and more complex, we used them to choose a number of representative strains (for each cluster with more than two strains with at least 90% similarity) for AFLP™.

Figure 4 RAPD PCR results using the DAF4 primer.

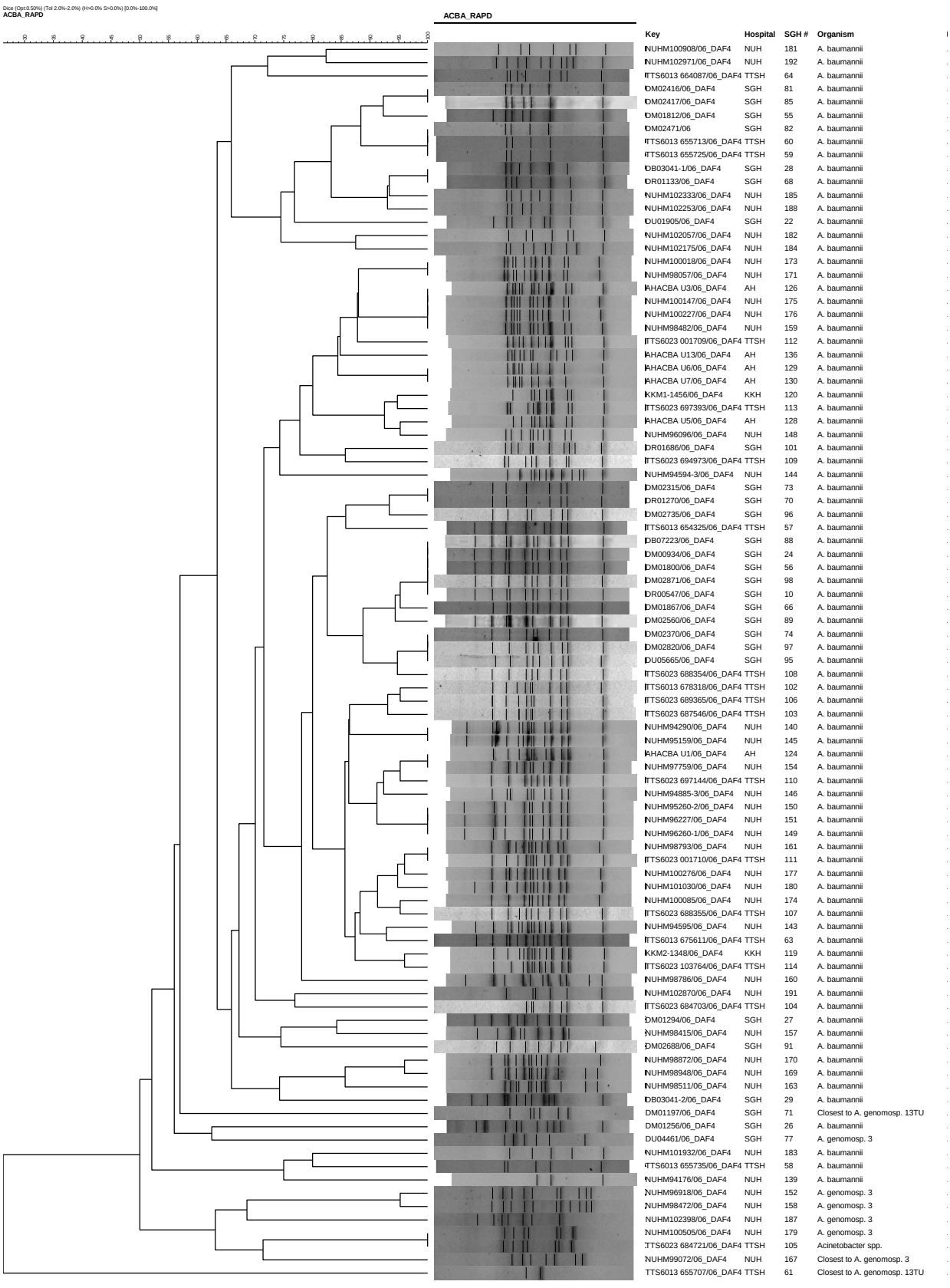
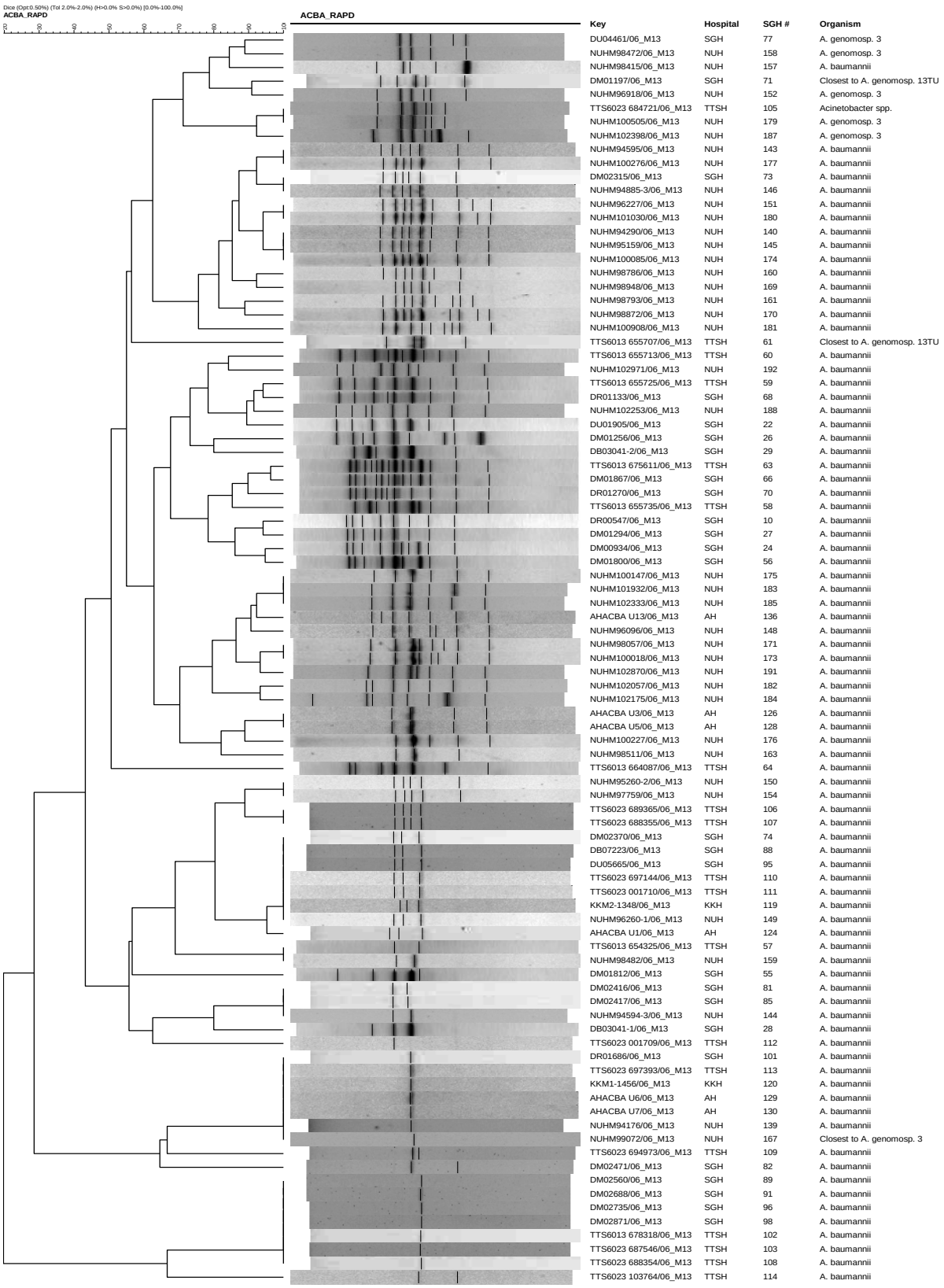


Figure 5 RAPD PCR results using the M13 primer.



AFLP™ analysis and MLST

Final identification of Acb to species by AFLP™ was in agreement with ITS sequencing or multiplex PCR identification. None of the included *A. pittii* and *A. nosocomialis* isolates clustered at 90% or above (Appendix 3). Among the *A. baumannii* isolates, the AFLP™ clustering pattern was more similar to that of RAPD PCR with the DAF4 primer than the M13 primer justifying the earlier decision to place more reliance on the DAF4 patterns. Three clusters of isolates grouped with ≥90% similarity (see Figure 6) indicating a high degree of genotypic relatedness.

DR25547 represented an outbreak of $bla_{\text{OXA-64}}$ containing *A. baumannii* isolated from hospital S in the eastern part of Singapore. This was the dominant outbreak strain in this hospital in 1996. DM09676 represented a cluster of $bla_{\text{OXA-88}}$ containing *A. baumannii* isolated from hospital S in the same year (61).

Comparison of AFLP™ profiles of the isolates to those of the Leiden database including strains identified to the European (International) clones I-III showed that 2 isolates clustered with clone I isolates at 80%, whereas 7 isolates were linked with clone II isolates at 78%. No isolate was found to cluster with clone III. All strains that clustered with International clones were resistant to imipenem and were positive for $bla_{\text{OXA-23-like}}$ with one exception (DU05665).

NUH M100227 (International clone I) was a representative of a cluster of isolates from hospitals A and N in the western part of Singapore in 2006.

DM18905 represented a cluster of *bla*$_{\text{OXA-69}}$ containing *A. baumannii* isolated from hospital S in 2001 (61). It also belonged to clone I, but was distinct from NUH M100227/06. Both were ST1 belonging to CC1 by MLST.

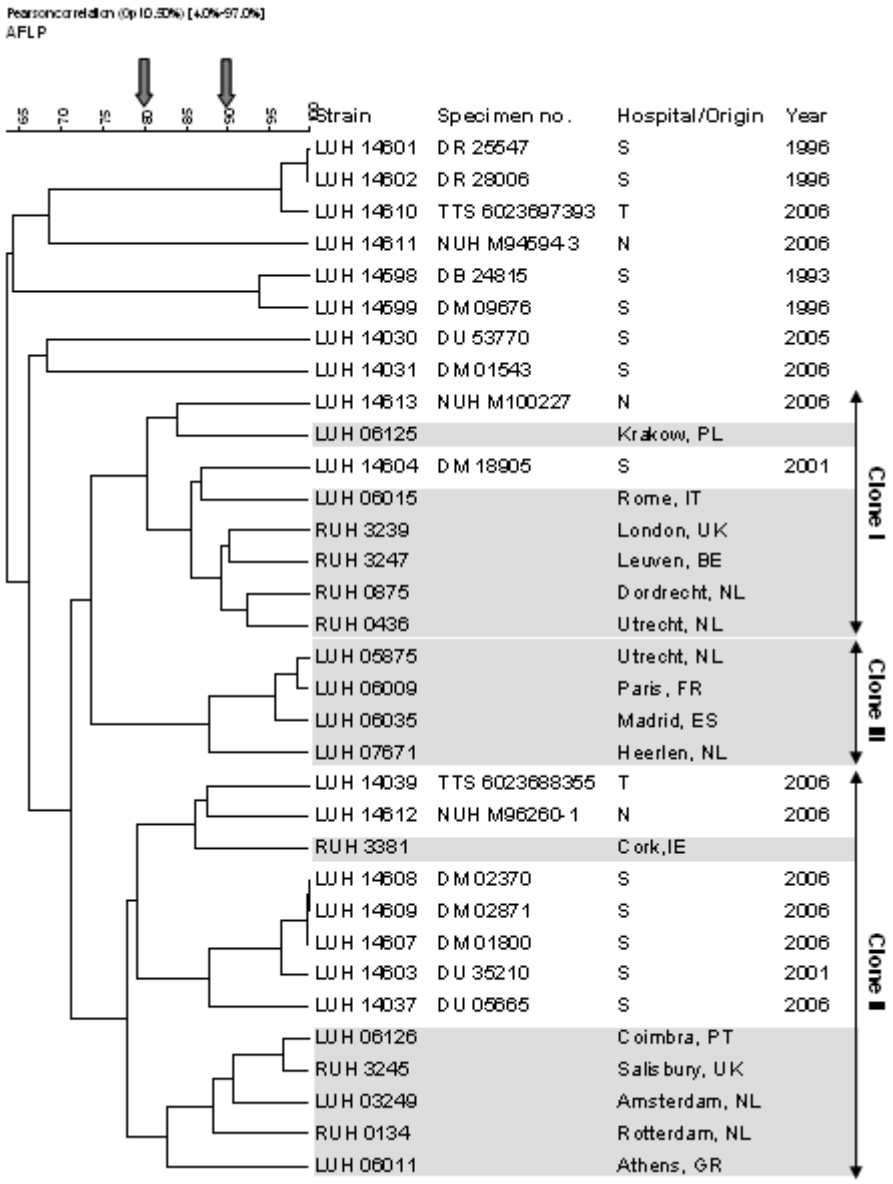
Of the seven isolates of the Singapore cluster linked with clone II at 78%, DM02370, DM02871, DM01800, and DU35210 were all isolated from hospital S, and represented a local clone that has persisted in this hospital from at least 2001 to 2006. DU35210 contained *bla*$_{\text{OXA-66}}$ and was part of the predominant outbreak type in 2001 (61). DU35210 was identified by MLST to ST2, CC2. Of the remaining isolates in clone II, DU05665 (*bla*$_{\text{OXA-23-like}}$ negative) was also isolated from hospital S. TTS 6023688355 and NUH M96260-1 were isolated from hospitals T and N respectively.

Assignment of isolates by AFLP™ to clones I and II correlated with assignment by MLST to ST1 and ST2 respectively. These two STs are the founders of the two largest clonal complexes in the total population eBURST analysis of *A. baumannii* (see Figure 7), emphasizing the global spread of these two clones which appear to be associated with *bla*$_{\text{OXA-23-like}}$ genes (93).

Six isolates, including the predominant outbreak strains from hospital S in 1996 that contained *bla*$_{\text{OXA-64}}$ had AFLP™ profiles that were unrelated to International clones I-III. Two isolates from 1996, 1 with *bla*$_{\text{OXA-64}}$ (DR25547) and the other with *bla*$_{\text{OXA-88}}$ (DM9679), were found to have MLST ST25 and novel type ST111 respectively. ST25 has been associated with *A. baumannii* in Greece, Italy and Turkey (94). Interestingly DR25547 (ST25) and DU35210

(ST2) in the original manuscript were classified in the same cluster by PFGE but the MLST data show that they were in fact unrelated in keeping with the difference in *bla*$_{\text{OXA-51-type}}$ genes (61). This illustrates the advantage of inferring relationships between strains using objective sequence-based typing methods like MLST over subjective band-based ones like PFGE.

Figure 6 Comparison of *A. baumannii* strains from Singapore with reference strains belonging to International clones I, II and III (marked in grey) by AFLP™. Arrows denote the similarity cut-off levels for strains (90%) and clones (80%).



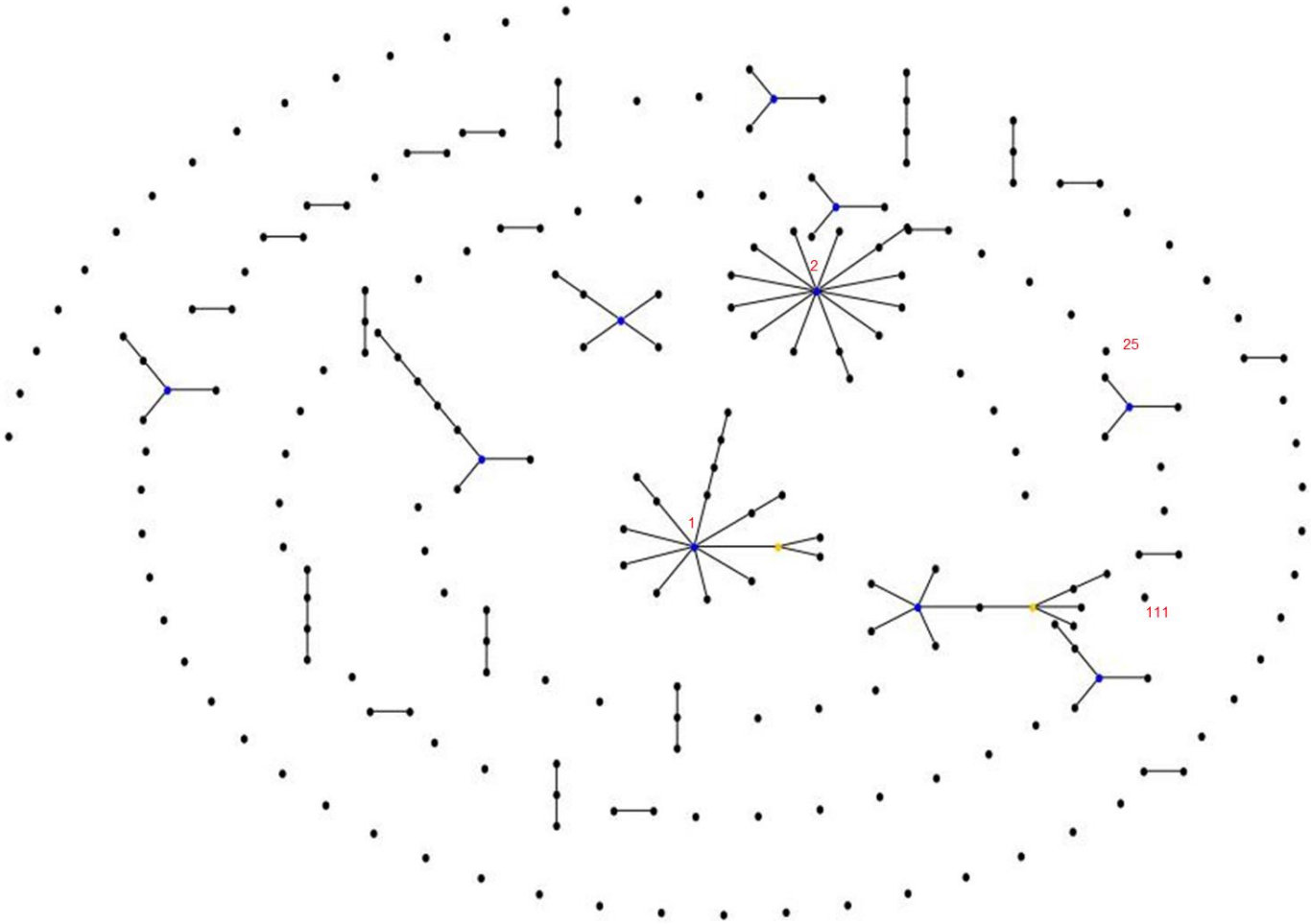


Figure 7 ‘Population Snapshot’ showing the clusters of linked and unlinked STs in the whole *A. baumannii* MLST database (19 January 2013). ST labels have been removed except for those of isolates from this study (in red). The predicted primary founder of a clonal complex is coloured blue and the subgroup founder is coloured yellow.

Discussion

Carbapenem resistance is increasing in Acb in Asia. The Chinese Meropenem Surveillance Study found that the prevalence of meropenem resistance in *Acinetobacter* spp. increased from 5.4% in 2003 to 39.3% in 2008 (95). Wang *et al* collected 221 imipenem-resistant *Acinetobacter* spp. from 11 hospitals in China from 1999 to 2005. One hundred and eighty-seven isolates were *A. baumannii*, 2 were *A. pittii*, and 5 were *A. nosocomialis*. Most isolates were associated with IS*Aba1-bla*$_{\text{OXA-23-like}}$, whereas no isolates were positive for IS*Aba1-bla*$_{\text{OXA-51-like}}$ (96).

Another study from China showed that carbapenem resistance was due to spread of clones that possessed IS*Aba1-bla*$_{\text{OXA-23-like}}$ (97). These isolates belonged to CC22 according to the MLST scheme of Bartual, and were closely related to International clone II (98). Carbapenem resistance in *A. baumannii* in nearby Hong Kong was likewise associated with IS*Aba1-bla*$_{\text{OXA-23-like}}$ in one study. However there did not seem to be any relationship to International clones based on comparison with the Leiden AFLP™ database (99).

Carbapenem-resistant *Acinetobacter* spp. has also increased in Korea. In a survey of over 35 hospitals in Korea, imipenem resistance in *Acinetobacter* spp. rose from 1% in 1997, to 13% in 2003, to 22% in 2007 (100, 101). Kim *et al* collected 190 isolates of *Acinetobacter* spp. in 2007 from 12 hospitals throughout Korea. 92.2% were *A. baumannii* of which 80% carried *bla*$_{\text{OXA-23-like}}$. IS*Aba1-bla*$_{\text{OXA-51-like}}$ was detected in 22 of the remaining isolates lacking

bla$_{\text{OXA-23-like}}$. Two *A. baumannii* and 1 *A. pittii* contained *bla*$_{\text{VIM-2}}$. Eight *A. pittii* and 1 *A. nosocomialis* contained *bla*$_{\text{IMP-1-like}}$ (102) . A single MLST clone ST22 belonging to CC22 was found to be responsible for the majority of imipenem-resistant *A. baumannii* in Korea (103). The prevalence of MBLs in *Acinetobacter* species may be higher in Korea compared to other countries. In one study, 26.5% of imipenem-resistant *Acinetobacter* spp. isolated from 2003-2004 in one hospital contained *bla*$_{\text{IMP-1-like}}$, *bla*$_{\text{VIM-2-like}}$ or *bla*$_{\text{SIM-1-like}}$ (104).

The mechanisms underlying carbapenem resistance may be quite different even for countries that are geographically quite close. In Taiwan, Lee *et al* collected 208 *Acinetobacter* species (*A. baumannii* 88.9%, *A. pittii* 4.8%, and *A. nosocomialis* 4.3%) from 4 hospitals from January 2001-March 2005. The imipenem resistance rates were 38.4% in *A. baumannii*, 70.0% in *A. pittii*, and 44.4% in *A. nosocomialis*. In *A. baumannii*, carbapenem resistance was most associated with IS*Aba1-bla*$_{\text{OXA-51-like}}$, followed by IS*1008-bla*$_{\text{OXA-58-like}}$, and *bla*$_{\text{OXA-24-like}}$. However, IS*Aba1-bla*$_{\text{OXA-51-like}}$ was also found in carbapenem-susceptible isolates. *bla*$_{\text{IMP-1}}$ was present in 2 isolates that also had IS*Aba-bla*$_{\text{OXA-58-like}}$. Surprisingly, unlike the situation in China and Korea, no *bla*$_{\text{OXA-23-like}}$ were found. Carbapenem resistance in *A. pittii* and *A. nosocomialis* were associated with IS*Aba3-bla*$_{\text{OXA-58-like}}$, though a few isolates had MBLs like *bla*$_{\text{IMP-1}}$ and *bla*$_{\text{IMP-8}}$ (105). These results were largely confirmed in a small study of multidrug-resistant *Acinetobacter* species in another hospital in Taiwan. *A. baumannii* comprised 70.7% of isolates, and *A. nosocomialis* and

A. pittii comprised 26.7%, and 2.7% respectively. Most (88%) carbapenem-resistant *A. baumannii* were associated with IS*Aba1-bla*$_{\text{OXA-51-like}}$ (106).

Alone among the Northeast Asian countries, Japan does not seem to have a major problem with carbapenem-resistant *Acinetobacter* spp. In a large multicenter survey of 100 hospitals in 2006, only 2.6% of 874 *Acinetobacter* spp. were resistant to imipenem (107). The reason for this discrepancy is unclear, there is a need to compare policies and perform molecular epidemiology studies among Asian countries to get more insight into these differences.

Surveillance of Acb in Southeast Asia has been less well documented using modern laboratory techniques. Multinational surveillance studies of the Asia-Pacific region exist but inevitably suffer from relatively small numbers that may not be representative compared to single country studies (108).

A survey of Acb in Singapore has previously been published based on isolates collected in 1996 and 2001 (61). However that study was confined to carbapenem-resistant isolates from one hospital only. Carbapenem resistance in *A. baumannii* was associated with *bla*$_{\text{OXA-23}}$, whereas carbapenem resistance in *A. pittii* and *A. nosocomialis* was associated with *bla*$_{\text{OXA-58}}$ and *bla*$_{\text{IMP-4}}$. At the time we did not test for the presence of insertion sequences preceding *bla*$_{\text{OXA-51-like}}$ genes in *A. baumannii*.

In the present study, almost 80% of Acb clinical isolates in Singapore were *A. baumannii* of which the majority were resistant to multiple antimicrobials. The majority of carbapenem-resistant *A. baumannii* appear to be associated with IS*Aba1*-*bla*$_{\text{OXA-23-like}}$. As in Korea and China, this seems related to the spread of International clones with outbreak potential. In hospital S, where archived isolates were available for study, International clones were only found from outbreak strains from 2001 and 2006 and were not represented among the outbreak strains from 1996. There seems to be a discrepancy in the *bla*$_{\text{OXA}}$ gene composition between the non-*A. baumannii* species. *bla*$_{\text{OXA-23-like}}$ was found in *A. nosocomialis* whereas *bla*$_{\text{OXA-58-like}}$ was predominantly in *A. pittii*.

Turton *et al* first suggested that IS*Aba1* located upstream of *bla*$_{\text{OXA51-like}}$ may contribute to carbapenem resistance in *A. baumannii* by acting as a promoter (109). This appears to be the situation in Taiwan but does not seem to be a major contributor to carbapenem resistance in *A. baumannii* in Singapore. Only 3 isolates of imipenem-resistant *A. baumannii* had IS*Aba1*-*bla*$_{\text{OXA-51-like}}$. These isolates still possessed *bla*$_{\text{OXA-23-like}}$ though this was not preceded by IS*Aba1*. One carbapenem-resistant *A. nosocomialis* had *bla*$_{\text{OXA-51-like}}$ that was not preceded by IS*Aba1*. Lee *et al* has recently described a *bla*$_{\text{OXA-51-like}}$ gene in *A. nosocomialis* (110). This is thought to have been acquired via a plasmid. It has been previously suggested that the presence of *bla*$_{\text{OXA-51-like}}$ genes can serve to identify *A. baumannii* (111). The presence of *bla*$_{\text{OXA-51-like}}$ in *A. nosocomialis* in this and the Taiwanese study (110) suggests that this may not be a sufficiently specific marker.

Only a few studies have defined the species distribution of Acb in clinical specimens (83). Consequently, most attention has been focused on *A. baumannii*. However, recently there has been increased appreciation of the clinical significance of other species in the Acb species complex. In our study, the relative prevalence *of A. nosocomialis* seems to be greater than that reported in other studies. In Hong Kong, *A. pittii* was more common than *A. baumannii* in blood cultures in 1997-1998 (112). In an Irish study, clinical isolates of *A. pittii* exceeded that of *A. baumannii* by a factor of 1.8. Carbapenem resistance in *A. pittii* (22%) also exceeded that of *A. baumannii* (4%) (113). In the Czech Republic, *A. nosocomialis* made up only 5.4% of isolates whereas *A. baumannii* (mostly clone II but also clone I) and *A. pittii* made up 73.5% and 20.4% respectively (114). *A. pittii* and *A. nosocomialis* isolates were susceptible to most antimicrobials tested including the carbapenems. Further, in an 8-year survey in a University hospital in the Netherlands, *A. pittii* (40.3% of Acb isolates) was second to *A. baumannii* (55.8%) whereas *A. nosocomialis* (3.9%) was much less common (83). The prevalence of multidrug-resistance in *A. pittii* ranged from 0% to 22% over the course of the study and no carbapenem-resistant isolates were detected.

In conclusion, *A. baumannii* was the predominant species within the Acb in clinical specimens in Singapore. Resistance was largely associated with IS*Aba1-bla*$_{\text{OXA-23-like}}$ and the strains that carried it belonged to the International clones which are notorious for their potential to spread. This situation is similar to that in China (97) and Korea (103). The relatively high rates of occurrence of *A. nosocomialis* and *A. pittii*, their presence in blood-stream

infections, and the multidrug and carbapenem resistance in these species underscore their potential clinical significance.

Chapter 4 Acquired Carbapenemases in *Enterobacteriaceae*

Introduction

In 1994, Osano *et al* described a plasmid-borne MBL gene bla_{IMP-1} in *S. marcescens* in Japan (33). A bla_{IMP-1}-positive *K. pneumonia*e (DB44384) was isolated in Singapore two years later (58). This was the first time a plasmid-borne carbapenemase had been found in any *Enterobacteriaceae* outside Japan. Since then, the Department of Pathology, Singapore General Hospital has actively looked out for Enterobactericeae with reduced susceptibility to carbapenems. In this manuscript, we describe our experience to date. DB44384 has been partially described before (115) and is included here, together with new typing and plasmid data for completeness.

Materials and methods

Isolates

All non-duplicate *Enterobacteriaceae* isolated from patient specimens received at the Department of Pathology, Singapore General Hospital that had reduced susceptibility (>1 µg/ml) to imipenem, or meropenem by Etest® (bioMérieux, Marcy l'Etoile, France) were archived for further testing. The identification of the isolates was confirmed by Vitek 2 (bioMérieux) or MALDI-TOF (Bruker, Bremen, Germany). Isolates were collected from October 1996 to May 2012.

Modified Hodge test

The isolates were tested by modified Hodge test (116). The methodology was as described in Chapter 2 except that 10 µg meropenem and ertapenem disks were used instead of imipenem.

Rosco KPC + MBL Confirm ID Test

Isolates that were modified Hodge test positive were tested with the KPC + MBL Confirm ID test (Rosco Diagnostica, Taastrup, Denmark). This is a commercial adaptation of the Imipenem-EDTA disk diffusion test described in Chapter 2. The test organism is inoculated onto a Mueller Hinton agar plate as previously described. One tablet each of the following is then applied to the inoculated agar; meropenem 10 µg, meropenem + dipicolinic acid (DP), meropenem + boronic acid (BO) and meropenem + cloxacillin (CX). After incubation overnight at 35ºC, the zones of inhibition around the tablets are measured. The meropenem + DP will show an increase in the inhibition zone diameter compared with the meropenem-only disk of ≥ 5 mm in the presence of a MBL. On the other hand the meropenem + BO will show an increase in the inhibition zone diameter compared with the meropenem-only disk of ≥ 5 mm in the presence of a KPC carbapenemase.

PCR for β-lactamase genes

Screening for β-lactamase genes associated with the carbapenemase-producer phenotype was performed by multiplex PCR to $bla_{\text{OXA-48-type}}$, bla_{KPC}, and MBL

genes (117). The primers for *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{SPM}$, *bla*$_{GIM}$, and *bla*$_{SIM}$ genes, were as previously described in Chapter 2. New primers are listed in the Table below. The primers were split between 3 multiplex PCRs performed separately. The first multiplex comprised *bla*$_{IMP}$, *bla*$_{VIM}$, and *bla*$_{SPM}$, the second comprised *bla*$_{NDM}$, *bla*$_{KPC}$, and *bla*$_{BIC}$, and the third comprised *bla*$_{AIM}$, *bla*$_{GIM}$, *bla*$_{SIM}$, and *bla*$_{DIM}$. PCR was carried out in a reaction volume of 50 µl with the QIAGEN multiplex PCR Kit (QIAGEN).

Table 10 Primers used in multiplex PCR for carbapenemase genes.

Gene	Name	Primer (5'-3')
bla$_{AIM}$	AIM-F	CTGAAGGTGTACGAAACAC
	AIM-R	GTTCGGCCACCTCGAATTG
bla $_{OXA-48}$	OXA-F	GCGTGGTTAAGGATGAACAC
	OXA-R	CATCAAGTTCAACCCAACCG
bla$_{BIC}$	BIC-F	TATGCAGCTCCTTTAAGGGC
	BIC-R	TCATTGGCGGTGCCGTACAC
bla$_{NDM}$	NDM-F	GGTTTGGCGATCTGGTTTTC
	NDM-R	CGGAATGGCTCATCACGATC
bla$_{DIM}$	DIM-F	GCTTGTCTTCGCTTGCTAACG
	DIM-R	CGTTCGGCTGGATTGATTTG
bla$_{KPC}$	KPC-Fm	CGTCTAGTTCTGCTGTCTTG
	KPC-Rm	CTTGTCATCCTTGTTAGGCG

The cycling conditions were: initial denaturation at 95°C for 15 min, followed by 36 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 40 s, and elongation at 72°C for 50 s, followed by a final extension at 72°C for 5 min.

PFGE

PFGE was performed in 1% agarose gels on total DNA extracted from *K. pneumoniae*, *E. coli*, and *E. cloacae* following restriction digestion with *Spe*I. For *K. pneumoniae* and *E. cloacae* the initial switch time was 0.5 s with a final switch time of 30 s using a linear ramp at 6 V/cm run for 20 h. For *E. coli*, the initial switch time was 5.3 s with a final switch time of 49.9 s using a linear ramp at 6 V/cm run for 20 hours. The PFGE band patterns were archived in Bionumerics (Applied Maths). However, PFGE pulsotypes and subtypes were designated according to Tenover's criteria (118). A representative of each distinct pulsotype was selected for further testing.

MLST

MLST was performed for *K. pneumoniae* using the Pasteur Institute protocol (119). This typing method is based on sequencing the following 7 house-keeping genes in the *K. pneumoniae* chromosome; *rpoB* (β-subunit of RNA polymerase), *gapA* (glyceraldehyde 3-phosphate dehydrogenase), *mdh* (malate dehydrogenase), *pgi* (phosphoglucose isomerase), *phoE* (phosphorine E), *infB* (translation initiation factor 2), and *tonB* (periplasmic energy transducer).

Table 11 Primers used for amplification of house-keeping genes for *K. pneumoniae* MLST.

Gene	Name	Primer (5'-3')
rpoB	rpoB:F:Vic3:oF	GTTTTCCCAGTCACGACGTTGTA **GGCGAAATGGCWGAGAACCA**
	rpoB:R:Vic2:oR	TTGTGAGCGGATAACAATTTC **GAGTCTTCGAAGTTGTAACC**
gapA	gapA:F:173:oF	GTTTTCCCAGTCACGACGTTGTA **TGAAATATGACTCCACTCACGG**
	gapA:R:181:oR	TTGTGAGCGGATAACAATTTC **CTTCAGAAGCGGCTTTGATGGCTT**
mdh	mdh:F:130:oF	GTTTTCCCAGTCACGACGTTGTA **CCCAACTCGCTTCAGGTTCAG**
	mdh:R:867:oR	TTGTGAGCGGATAACAATTTC **CCGTTTTTCCCCAGCAGCAG**
pgi	pgi:F:1R:oF	GTTTTCCCAGTCACGACGTTGTA **GAGAAAAACCTGCCTGTACTGCTGGC**
	pgi:R :1F:oR	TTGTGAGCGGATAACAATTTC **CGCGCCACGCTTTATAGCGGTTAAT**
phoE	phoE:F:604.1:oF	GTTTTCCCAGTCACGACGTTGTA **ACCTACCGCAACACCGACTTCTTCGG**
	phoE:R:604.2:oR	TTGTGAGCGGATAACAATTTC **TGATCAGAACTGGTAGGTGAT**
infB	infB:1F:oF	GTTTTCCCAGTCACGACGTTGTA **CTCGCTGCTGGACTATATTCG**
	infB:1R:oR	TTGTGAGCGGATAACAATTTC **CGCTTTCAGCTCAAGAACTTC**
tonB	tonB:1F:oF	GTTTTCCCAGTCACGACGTTGTA **CTTTATACCTCGGTACATCAGGTT**
	tonB:2R:oR	TTGTGAGCGGATAACAATTTC **ATTCGCCGGCTGRGCRGAGAG**

The annealing temperature for all primers was 50ºC. Sequencing was performed in both directions with the universal primers; primer oF (5'-GTTTTCCCAGTCACGACGTTGTA-3') and primer oR (5'-TTGTGAGCGGATAACAATTTC-3'). MLST profiles were submitted to eBURST V3 (http://eburst.mlst.net/) on 19 January 2013. Isolates sharing six out of seven alleles were assigned to the same BURST group and were considered to belong to the same clonal complex descended from a common founder genotype.

MLST was performed on *E. coli* using the University of Cork protocol (120). This typing method is based on sequencing the following seven house-keeping genes in the *E. coli* chromosome; *adK* (adenylate kinase), *fumC* (fumarate hydratase), *gyrB* (DNA gyrase), *icd* (isocitrate/isopropylmalate dehydrogenase), *mdh* (malate dehydrogenase), *purA* (adenylosuccinate dehydrogenase), and *recA* (ATP/GTP binding motif).

Table 12 Primers used for amplification of house-keeping genes for *E. coli* MLST.

Gene	Name	Primer (5'-3')
adK	adkF	ATTCTGCTTGGCGCTCCGGG
	adkR	CCGTCAACTTTCGCGTATTT
fumC	fumCF	TCACAGGTCGCCAGCGCTTC
	fumCR	GTACGCAGCGAAAAAGATTC
gyrB	gyrBF	TCGGCGACACGGATGACGGC

	gyrBR	ATCAGGCCTTCACGCGCATC
icd	icdF	ATGGAAAGTAAAGTAGTTGT TCCGGCACA
	icdR	GGACGCAGCAGGATCTGTT
mdh	mdhF	ATGAAAGTCGCAGTCCTCGG CGCTGCTGGCGG
	mdhR	TTAACGAACTCCTGCCCCAGA GCGATATCTTTCTT
purA	purAF	CGCGCTGATGAAAGAGATGA
	purAR	CATACGGTAAGCCACGCAGA
recA	recAF	CGCATTCGCTTTACCCTGACC
	recAR	TCGTCGAAATCTACGGACCGGA

The cycling conditions were: initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at the appropriate temperature for 1 min, and elongation at 72°C for 2 min, followed by a final extension at 72°C for 5 min. The annealing temperatures were 54ºC for *adk*, *fumC*, *icd*, and *purA*, 58ºC for *recA* and 60ºC for *gyrB* and *mdh*. Sequencing was performed in both directions with the same primers used for amplification. MLST profiles were submitted to eBURST V3 (http://eburst.mlst.net/) on 19 January 2013. Isolates sharing six out of seven alleles were assigned to the same BURST group and were considered to belong to the same clonal complex descended from a common founder genotype.

Sequencing of carbapenemase genes and their flanking regions

Amplification and sequencing of *bla*$_{IMP}$, *bla*$_{NDM}$, and *bla*$_{KPC,}$ and their flanking regions was performed using previously published protocols (76, 121-123). For the flanking regions, PCR was carried out using an internal primer for the carbapenemase gene and the appropriate primer for the flanking region.

Table 13 Primers used for amplification of the flanking regions of *bla*$_{IMP}$.

Gene	Name	Primer (5'-3')
Integron	INT-5'CS-f	CTTCTAGAAAACCGAGGATGC
	INT-3'CS-r	CTCTCTAGATTTTAATGCGGATG
bla$_{IMP}$	IMP1ASF	TGAGCAAGTTATCTGTATTC
	IMP1ASR	TTAGTTGCTTGGTTTTGATG
	IMP1SF	CGGGCCTGGATAAAAAACTT
	IMP1SR	GCCACAAATTCATTTAGCGG

For the flanking regions of *bla*$_{IMP}$, the cycling conditions were 35 cycles of denaturation at 94°C for 1 min, annealing at the 55°C for 1 min, and elongation at 72°C for 5 min (with an increment of 5 s for each cycle).

Table 14 Primers used for amplification of flanking regions of *bla*$_{NDM}$.

Gene	Name	Primer (5'-3')
IS*Aba125*	ISAba125ext	ACACCATTAGAGAAATTTGC
ble$_{MBL}$	bleo-Rev	GGCGATGACAGCATCATCCG

For the flanking regions of *bla*$_{NDM}$, flanking primers were used together with NDM-F and NDM-R. The cycling conditions were 95°C for 15 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and elongation at 72°C for 2 min, followed by a final extension at 72°C for 7 min.

Table 15 Primers used for amplification of flanking regions of *bla*$_{KPC}$.

Name	Primer (5'-3')
For5451	TGGCCAGGATGTACAACGTC
Kpc-dw	ACAGTGGTTGGTAATCCATGC
For5958	TCAAGCTTCTGACCGACAAC
Re6838	CCTTGAATGAGCTGCACAGT
Re8069	TCCGTAGTGAGGCTGTTCTG
Kpc-up	GCTACACCTAGCTCCACCTTC
Re8728	CGAACATAAGGCCGAACGTG
For7085	GCGATACCACGTTCCGTCTG
For7755	ACAGATACGCCATTCGCCTC

For the flanking regions of *bla*$_{KPC}$, the cycling conditions were 95°C for 15 minutes, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and elongation at 72°C for 2.5 min, followed by a final extension at 72°C for 7 min.

Table 16 Primers used for amplification of *bla*$_{OXA-48}$, *bla*$_{OXA-181}$ and their flanking regions.

Gene	Name	Primer (5'-3')
bla$_{OXA-181}$	OXA181ASF	ATGCGTGTATTAGCCTTATCGG
	OXA181ASR	CTAGGGAATAATTTTCTCCTGTTTG
bla$_{OXA-181}$ flanking region	OXA181ISEcpI-F	TTCAAAATGATGATCCCCTCGTC
	OXA181repA-R	GCCTTGTTCGTGACGGTAAT
bla$_{OXA-48}$ flanking region	IS1999F	TAATAAATATCTGCAGTTTATGCTGGT
	IS1999R	ATGTCGAATCGCTCTGGACT

In-house primers were designed using Primer3 (http://primer3.wi.mit.edu) for *bla*$_{OXA-48}$ and *bla*$_{OXA-181}$ and their flanking regions based on sequences in GenBank (accession numbers JN626286.1 and JN205800.1). For amplification of the *bla*$_{OXA-48}$ and *bla*$_{OXA-181}$ genes, the cycling conditions were 95°C for 15 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 44°C for 30 s, and elongation at 72°C for 1 min, followed by a final extension at 72°C for 7 min.

Conjugation

Conjugation of plasmids was attempted by simple plate mating. Donor (carbapenemase-producing isolates) and recipient (*E. coli* K12 J53 resistant to azide or *E. coli* K12 J53 resistant to rifampicin) were incubated on Luria-

Bertani (LB) agar (BD), overnight at 35ºC in air. The following day, 1-3 colonies were inoculated into 5 ml of LB broth (BD) and incubated overnight at 35ºC in air in a Heidolph Promax 1020 shaking incubator (Heidolph Instruments GmbH & Co, Schwabach, Germany). After incubation, 500 µl of donor and 500 µl of recipient were mixed and 100 µl of the mixture inoculated onto LB agar (BD). After overnight incubation in air at 35ºC, all the growth on the plate was suspended in 5 ml of normal saline with a swab. The suspension was vortexed and 100 µl inoculated onto MacConkey agar (Oxoid Ltd, Basingstoke, UK) with added meropenem (Sigma, St. Louis, MO, USA) 1-2 µg/ml and sodium azide (Sigma) 100 µg/ml or rifampicin (Sigma) 200 µg/ml as appropriate. The plates were incubated in air for up to 5 days at 35ºC. The plates were inspected daily for *E. coli* transconjugants and up to 5 suspect colonies were subcultured for further testing and archiving. The identity of the transconjugants was confirmed by a positive spot indole test and decreased susceptibility to a panel of β-lactam antibiotics including carbapenems. DNA extraction was performed by boiling a suspension of the transconjugants in molecular grade water for 15 min.

Chemical transformation

Isolates that failed to conjugate underwent chemical transformation. Plasmid DNA was extracted from carbapenemase-producing isolates using QIAprep Spin Miniprep Kits (Qiagen).

Chemical transformation was performed using TaKaRa *E. coli* DH5α competent cells (TaKaRa, Shiga, Japan), or BL21 (DE3) Competent Cells (Stratagene, La Jolla, CA, USA). The competent cells were first thawed on ice. Plasmid extract (5-10 µl) was added to 100 µl competent cells in an ice-cold eppendorf tube and mixed by pipetting. After incubating on ice for 30 min, the mixture was subjected to heat shock at 42ºC for 45 s before quenching on ice for 2 min. LB broth (900 µl) was added and the entire mixture was incubated for 45-60 min at 35ºC in a shaking incubator. The cells were pelleted by centrifuging at 5,000 rpm for 5 min and the cells spread onto LB agar (BD) containing meropenem (Sigma) 1 µg/ml or ampicillin (Sigma) 50 µg/ml.

Transformation by electroporation

For plasmids that failed chemical transformation, electroporation was attempted using One Shot® TOP10 Electrocomp™ cells (Life Technologies, NY, USA). The cells (40 µl) were thawed on ice before adding to 1-2 µl of pre-chilled plasmid extract in a microcentrifuge tube on ice and mixed gently without pipetting. The cells were then left on ice for 1 min. The cells were then transferred to a chilled cuvette and pulsed in a MicroPulsar Electroporator (BIO-RAD). SOC medium (1 ml) was added immediately and the cells resuspended with a Pasteur pipette. The cell suspension was transferred to a polypropylene tube and incubated at 35ºC for 60 min with shaking. After incubation, the cells spread onto LB agar (BD) containing

meropenem (Sigma) 1 µg/ml or ampicillin (Sigma) 50 µg/ml. Successful transformants were processed the same way as transconjugants.

PCR based plasmid replicon typing

The PCR based plasmid replicon typing method of Carattoli *et al* was performed on DNA extracts of successful transconjugants and transformants using the PBRT kit (DIATHEVA, Fano, Italy) (124). This kit comprises a set of 8 multiplex PCRs for the amplification of 25 replicons that represent the major plasmid incompatibility groups and replicase genes found in resistance plasmids circulating in *Enterobaceriaceae.*

PCR labelling of DNA probes to carbapenemase genes

PCR labelling of DNA probes was done using the PCR DIG Probe synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany). The DNA templates were extracts from isolates DU9800 ($bla_{\text{IMP-1}}$), DM6277 ($bla_{\text{KPC-2}}$), DS8293 ($bla_{\text{NDM-1}}$), DB4758 ($bla_{\text{OXA-48}}$). The corresponding primer pairs that had been used for multiplex PCR detection of carbapenemase genes (117) were used to generate each probe with the exception of bla_{IMP} where we used the primers described by Yan *et al* instead (76).

Table 17 Primers used to generate DNA probes to carbapenemase genes.

Gene	Name	Primer (5'-3')
bla$_{IMP}$	IMP-1ASF	TGAGCAAGTTATCTGTATTC
	IMP-1ASR	TTAGTTGCTTGGTTTTGATG
bla $_{KPC-2}$	KPC-Fm	CGTCTAGTTCTGCTGTCTTG
	KPC-Rm	CTTGTCATCCTTGTTAGGCG
bla$_{NDM-1}$	NDM-F	GGTTTGGCGATCTGGTTTTC
	NDM-R	CGGAATGGCTCATCACGATC
bla$_{OXA-48}$	OXA-F	GCGTGGTTAAGGATGAACAC
	OXA-R	CATCAAGTTCAACCCAACCG

For each labelled probe, a separate unlabelled probe was made to evaluate probe labelling. The cycling conditions were: initial denaturation at 95ºC for 2 min, followed by 30 cycles of denaturation at 95ºC for 30 s, annealing at 60ºC for 30 s, and elongation at 72ºC for 40 s followed by a final elongation step at 72ºC for 7 min. The probes were evaluated by running the PCR products in a 2% TBE gel. The labelled PCR products had a higher molecular weight than the unlabelled products because of the high density labelling with DIG.

S1 nuclease digestion of plasmids carrying carbapenemase genes

DNA extracts from successful transconjugants, transformants, and strains that were unable to transfer the carbapenemase gene were prepared in agarose plugs and digested with 10U of S1 nuclease in 150 µl of buffer using a method adapted from that described by Barton *et al* (125). The digested products were

run in a 1% TBE gel using an initial switch time of 5.3 seconds and a final switch time of 34.9 seconds with a linear ramp at 6 V/cm run for 20 hours.

Southern blotting of S1 nuclease digested plasmids carrying carbapenemase genes

The gels were first depurinated in 250 mM HCl with shaking at room temperature for 20 min. The DNA in the gels was then denatured by submerging in Denaturation Solution (0.5 M NaoOH, 1.5 M NaCl) for 2 X 15 min at room temperature with gentle shaking. After rinsing with sterile, double distilled water, the gels were submerged in Neutralization Solution (0.5 M Tris-HCl, pH 7.5; 1.5 M NaCl) for 2 X 15 min at room temperature. The gels were then equilibrated for at least 10 min in 20X SSC (3 M NaCl, 300 mM sodium citrate, pH 7.0).

The blot transfers were set up in an empty Sub Cell electrophoresis tank (BIO-RAD) by first placing a piece of Whatman 3MM paper to act as a bridge between two separate reservoirs containing 20X SSC. A gel-sized piece of Whatman 3MM paper soaked in 20X SSC was placed on top of the bridge and the gel was then placed upon this with surface where the gel plugs were inserted facing the paper (i.e. the gel is flipped over from its orientation during electrophoresis). A gel-sized piece of positively charged GeneScreen Plus® nylon hybridization transfer membranes (NEN™ Life Science Products, Boston, USA) was placed on the DNA containing surface of the gel. A dry

gel-sized piece of Whatman 3MM paper was placed on the membrane followed by a stack of paper towels, a glass plate and a weight of approximately 400 g. The blot was allowed to transfer overnight in 20X SSC.

After DNA transfer, the membranes were placed DNA side facing up on Whatman 3MM paper that had been soaked in 2X SSC in a CL-1000 Ultraviolet Crosslinker (UVP, Upland, CA, USA). The wet membrane was exposed to UV light for 3 min and rinsed briefly in sterile, double-distilled water before being left to air dry.

DNA hybridization of probes onto Southern blots

Pre-hybridization and hybridization were performed in sealable plastic boxes in a Shake 'n' Stack hybridization oven (Thermo Electron Corporation, Marietta, OH, USA). The hybridization temperatures were 45ºC for bla_{IMP}, 52ºC for bla_{NDM}, 46ºC for bla_{OXA}, and 52ºC for bla_{KPC}. The membranes were first incubated in pre-warmed pre-hybridization buffer-DIG Easy Hyb (Roche) for 30 min. The appropriate labelled probe (2 µl of labelled probe per ml of hybridization buffer) was placed in a microcentrifuge tube containing 50 µl of water and boiled for 5 min to denature the probe. The denatured probe was immediately chilled in ice and added to pre-warmed DIG Easy Hyb (3.5 ml per 100 cm^2 of membrane) and mixed by inversion to form the hybridization solution. The pre-hybridization buffer was discarded and immediately

replaced with hybridization solution. The blots were incubated overnight at the appropriate hybridization temperature.

After hybridization, the membranes were removed from the hybridization solution and placed in a plastic tray and washed twice for 5 min each with Low Stringency buffer (2X SSC containing 0.1% SDS) at room temperature with shaking. The Low Stringency buffer was then replaced and the membranes washed twice at the appropriate wash temperature for 15 min each with preheated High Stringency Buffer (0.5X SSC + 0.1% SDS at 65ºC for bla_{IMP}, 0.1X SSC + 0.1% SDS at 68ºC for bla_{NDM}, bla_{OXA}, and bla_{KPC}).

Visualizing the hybridization reaction

The membranes were washed for 2 min at room temperature with shaking in a plastic container containing Washing Buffer (0.1 M Maleic acid, 0.15 M NaCl; pH 7.5; 0.3% Tween 20). The Washing Buffer was then discarded and the membranes incubated in freshly prepared Blocking Solution (Roche) for at least 30 min with shaking. The Blocking Solution was then discarded and the membranes incubated with Antibody Solution (Roche) containing Anti-digoxigenin-AP diluted in Blocking Solution for 30 min. The antibody solution was discarded and the membranes washed twice for 15 min each with Washing Buffer. The membranes were then equilibrated for 3 min in Detection Buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5).

Each membrane was covered completely with Color Substrate Solution made from NBT/BCIP stock solution (Roche) and incubated overnight in the dark without shaking. The colour reaction was stopped by rinsing the membrane for 5 min in TE buffer and the image digitally archived using a Gel Doc™ 2000 (BIO-RAD).

Plasmid fingerprinting

Plasmid DNA was extracted from successful transconjugants and transformants using QIAprep Spin Miniprep Kits (Qiagen). Extracts of plasmids that were of the same size by S1 nuclease digestion were digested with 20U of *Hind*III and *Pst*I overnight and run in 2% agarose gels. Plasmids with the same restriction digest patterns were assigned to the same plasmid type.

Results

During the study period, we collected 41 isolates of *Enterobacteriaciae* that produced carbapenemases from 40 patients. The majority were *K. pneumoniae* (24 isolates), followed by *E. coli* (8 isolates), *Enteroacter cloacae* (7 isolates), and *Citrobacter* spp. All isolates with the exception of 3 were isolated from patients in the Singapore General Hospital with the remainder referred from private hospitals. Twelve patients were foreigners and the rest were locals.

The ROSCO KPC + MBL Confirm ID Test correctly predicted the presence of MBLs in *bla*$_{NDM}$ and *bla*$_{IMP}$ positive isolates. *bla*$_{KPC-2}$ positive isolates were also accurately predicted. Isolates that were modified Hodge test positive but negative for the ROSCO KPC + MBL Confirm ID Test were found to have either *bla*$_{OXA-48}$ or *bla*$_{OXA-181}$.

We succeeded in conjugating or transforming all plasmids bearing carbapenemase genes with the exception of those bearing *bla*$_{IMP}$ in *K. pneumoniae* DM23092, DS6941, and *E. cloacae* DM9800, *bla*$_{NDM-1}$ in *K. pneumoniae* DR40294, and all those with *bla*$_{OXA-181}$. The results are summarized in Table 18.

Table 18 Characteristics of carbapenemase-producing *Enterobacteriaceae*.

Species	Isolate	Site	Year	Nationality	Carbapenemase	MIC (mg L^{-1})			PFGE	ST	Plasmid		
						IMP	MEM	ETP			Inc	Size (kb)	Type
K. pneumoniae	DB44384	Blood	1996	SIN	IMP-1	>32	>32	>32	dg	42	A/C	c.a. 160	3
K. pneumoniae	DU32157	Urine	2011	SIN	IMP-1	4	4	16	KPN9	147	A/C	c.a. 150	3
K. pneumoniae	DR37041	Resp	2010	SIN	IMP-1	>32	>32	>32	KPN4	885	A/C	c.a. 160	3
C. freundii	DU10513	Urine	2012	SIN	IMP-1	4	8	16	nd	na	A/C	c.a. 150	3
E. cloacae	DU31899	Urine	2011	SIN	IMP-1	1	1	4	ECL1	na	A/C	c.a. 190	nt
E. cloacae	DM9800	Wound	2011	SIN	IMP-1	2	4	>32	ECL2	na	nt	c.a. 270	nt
K. pneumoniae	DM23092	Wound	2004	BAN	IMP-1	3	3	16	KPN1	11	nt	nt	nt
K. pneumoniae	DS6941	Stool	2010	SIN	IMP-4	>32	>32	>32	KPN6	568	nt	c.a. 150	nt
E. cloacae	DM8861	CVP tip	2012	SIN	KPC-2	8	4	8	ECL7	na	neg	c.a. 50	2
E. coli	DM6277	Wound	2012	SIN	KPC-2	2	2	8	ECO7	3054	neg	c.a. 50	2
K. pneumoniae	DR2160	Resp	2012	SIN	KPC-2	16	32	16	KPN13	841	neg	c.a. 50	2
K. pneumoniae	DU51131	Urine	2011	SIN	KPC-2	>32	8	>32	KPN11	11	neg	c.a. 50	2
K. pneumoniae	DB2244	Blood	2012	SIN	KPC-2	>32	>32	>32	KPN12	11	FIIK	c.a. 100	nd

K. pneumoniae	DU1301	Urine	2010	SIN	NDM-1	>32	>32	>32	KPN2	11	neg	c.a. 80	10
K. pneumoniae	DU52392	Urine	2011	SIN	NDM-1	8	8	>32	KPN10	11	neg	c.a. 80	11
C. sediakii	DM5680-1	Tissue	2012	SIN	NDM-1	32	32	32	nd	na	neg	c.a. 90	12
E. cloacae	DM5887-3	Wound	2012	SIN	NDM-1	>32	>32	>32	ECL6	na	R	c.a. 110	13
K. pneumoniae	DU1883	Urine	2011	SIN	NDM-1	>32	>32	>32	KPN8	147	neg	c.a. 120	14
K. pneumoniae	DM3906	Abdo Fluid	2012	SIN	NDM-1	>32	>32	>32	KPN8-1	147	FIIK	c.a. 160	nd
E. coli	DS8293	Stool	2010	SIN	NDM-1	>32	>32	>32	ECO1	2083	FII	c.a. 110	15
E. coli	DU48916	Urine	2011	IND	NDM-1	>32	>32	>32	dg	405	A/C	c.a. 150	16
E. cloacae	DB6217	Blood	2012	RIN	NDM-1	16	>32	>32	ECL5	na	A/C	c.a. 150	16
E. cloacae	DM16303	Wound	2011	SIN	NDM-1	>32	32	>32	ECL4	na	neg	c.a. 40	4
K. pneumoniae	DU43320	Urine	2010	SIN	NDM-1	>32	8	>32	KPN5	273	neg	c.a. 40	4
K. pneumoniae	DR2834	Resp	2011	MAL	NDM-1	>32	>32	>32	KPN5-1	273	neg	c.a. 40	4
K. pneumoniae	DS1731	Stool	2011	SIN	NDM-1	>32	>32	>32	KPN5	273	neg	c.a. 40	4
E. coli	DS205	Stool	2011	BAN	NDM-1	>32	>32	>32	ECO2	648	neg	c.a. 40	5
E. cloacae	DM15118	Wound	2011	VIE	NDM-1	>32	>32	>32	ECL3	na	neg	c.a. 60	8
E. coli	DS474	Stool	2011	SIN	NDM-1	>32	>32	>32	ECO3	101	FII	c.a. 60	9

E. coli	DS1878	Stool	2011	SIN	NDM-1	2	4	>32	ECO4	2451	A/C	c.a. 370	nd
K. pneumoniae	DS159	Stool	2011	RIN	NDM-1	>32	>32	>32	KPN8	nd	nd	nd	nd
K. pneumoniae	DU44951	Urine	2010	VIE	NDM-1	>32	>32	>32	KPN7	1	nt	c.a. 40	nt
E. coli	DM20217	Abdo fluid	2011	SIN	NDM-7	>32	>32	>32	ECO5	205	neg	c.a. 40	6
K. pneumoniae	DU7433	Urine	2010	BAN	NDM-1	>32	>32	>32	KPN3	14	A/C	c.a. 60	7
K. pneumoniae	DR40294	Resp	2011	SIN	NDM-1	>32	>32	>32	KPN3-1	14	nt	c.a. 280	nt
K. pneumoniae	DB53879	Blood	2011	BAN	OXA-181	32	>32	>32	KPN3-1	nd	nt	c.a. 150	nt
K. pneumoniae	DX1083	Resp	2011	BAN	OXA-181	>32	>32	>32	KPN3-1	14	nt	c.a. 150	nt
K. pneumoniae	DU54621	Urine	2011	BAN	OXA-181	>32	>32	>32	KPN3-1	nd	nt	c.a. 150	nt
K. pneumoniae	R16-09	Resp	2012	SIN	OXA-181	>32	>32	>32	KPN3-1	nd	nt	c.a. 150	nt
E. coli	DB4758	Blood	2012	SIN	OXA-48	>32	32	>32	ECO6	2003	neg	c.a. 50	1
K. pneumoniae	DU20470-1	Urine	2012	SIN	OXA-48	4	1	8	KPN14	29	neg	c.a. 50	1

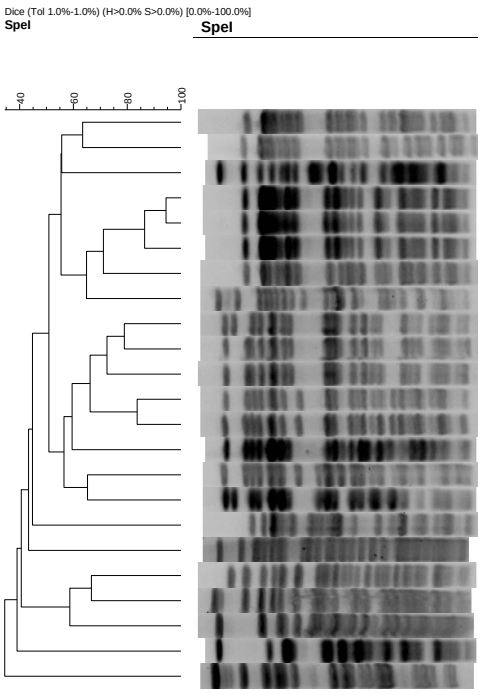
dg, degraded; nt, not transferred; nd, not done; na, not applicable; SIN, Singapore; BAN, Bangladesh; VIE, Vietnam;

RIN, Indonesia; MAL, Malaysia; IND, India.

PFGE

Apart from 7 *K. pneumoniae* (DS159, DU1883 and DM3906 belonging to PFGE type KPN8, DR2834, DU43320 and DS1731 belonging to KPN5, DU7433 and DR40294 belonging to KPN3), the isolates with $bla_{\text{NDM-1}}$ had distinctive PFGE patterns. The 3 *K. pneumoniae* with $bla_{\text{KPC-2}}$ had distinct PFGE patterns whereas isolates with $bla_{\text{OXA-181}}$ were clonal. Surprisingly, $bla_{\text{NDM-1}}$ bearing *K. pneumoniae* DU7433 and DR40294 had similar PFGE patterns to *K. pneumoniae* with $bla_{\text{OXA-181}}$. These isolates all belonged to ST14 which may be a common clone in Bangladesh as the majority were isolated from patients of Bangladeshi origin. *E. coli* and *E. cloacae* with carbapenemase genes were not clonal.

Figure 8 PFGE patterns of *K. pneumoniae* isolates with year of isolation, strain number, carbapenemase and MLST sequence type.



.2010	DU7433	NDM-1	ST14
.2012	R16-09	OXA-181	
.2004	DM23092	IMP-1	ST11
.2011	DB53879	OXA-181	
.2012	DU54621	OXA-181	
.2011	DX1083	OXA-181	ST14
.2011	DR40294	NDM-1	ST14
.2010	DU44951	NDM-1	ST1
.2011	DR2834	NDM-1	ST273
.2011	DS1731	NDM-1	ST273
.2010	DU43320	NDM-1	ST273
.2011	DS159	NDM-1	
.2011	DU1883	NDM-1	ST147
.2010	DR37041	IMP-1	ST885
.2012	DM3906	NDM-1	ST147
.2011	DU32157	IMP-1	ST147
.2011	DU52392	NDM-1	ST11
.2012	DU20470-1	OXA-48	ST29
.2010	DU1301	NDM-1	ST11
.2011	DU51131	KPC-2	ST11
.2012	DB2244	KPC-2	ST11
.2010	DS6941	IMP-4	ST568
.2012	DR2160	KPC-2	ST841

Figure 9 PFGE patterns of *E. coli* isolates with year of isolation, strain number, carbapenemase and MLST sequence type.

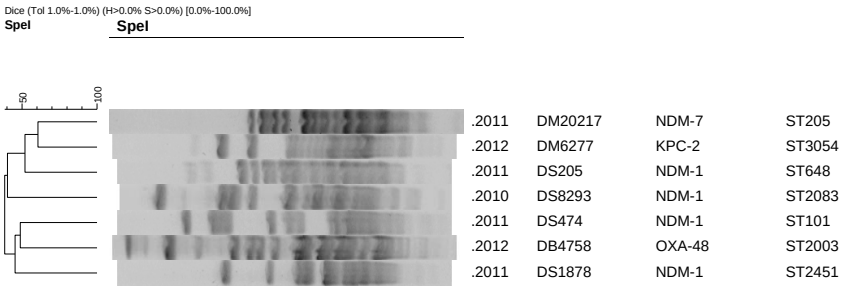
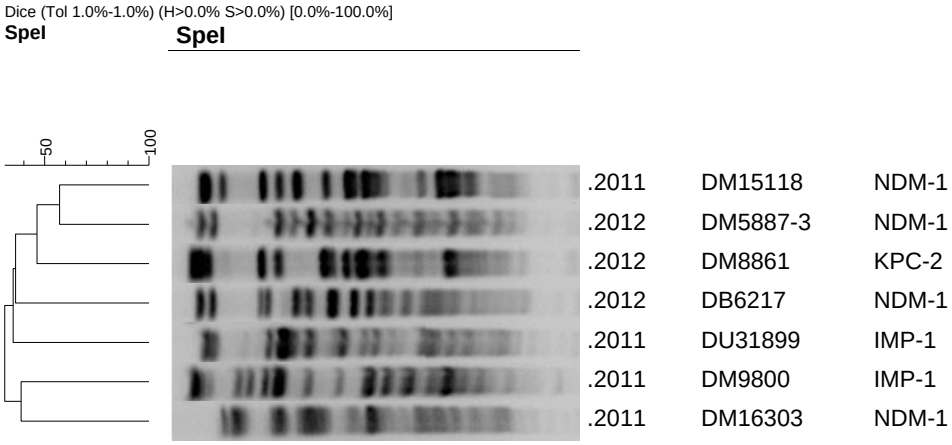


Figure 10 PFGE patterns of *E. cloacae* isolates with year of isolation, strain number and carbapenemase.



MLST

The MLST results for *K. pneumoniae* and *E. coli* are summarized in Table 18. The results correlated well with the PFGE results. For *K. pneumoniae*, ST11 and ST14 were the most common whereas the *E. coli* STs were all different. The evolutionary relationship of the isolates from this study to each other relative to the whole *K. pneumoniae* and *E. coli* MLST databases are shown in the figures below.

For *K. pneumoniae*, ST42 and ST29 are founders of different clonal complexes. ST11 and ST14 come from the same clonal complex. ST147 is the founder of a clonal complex that includes ST885 and ST273.

For *E. coli*, ST101, ST405 and ST648 are founders of different clonal complexes.

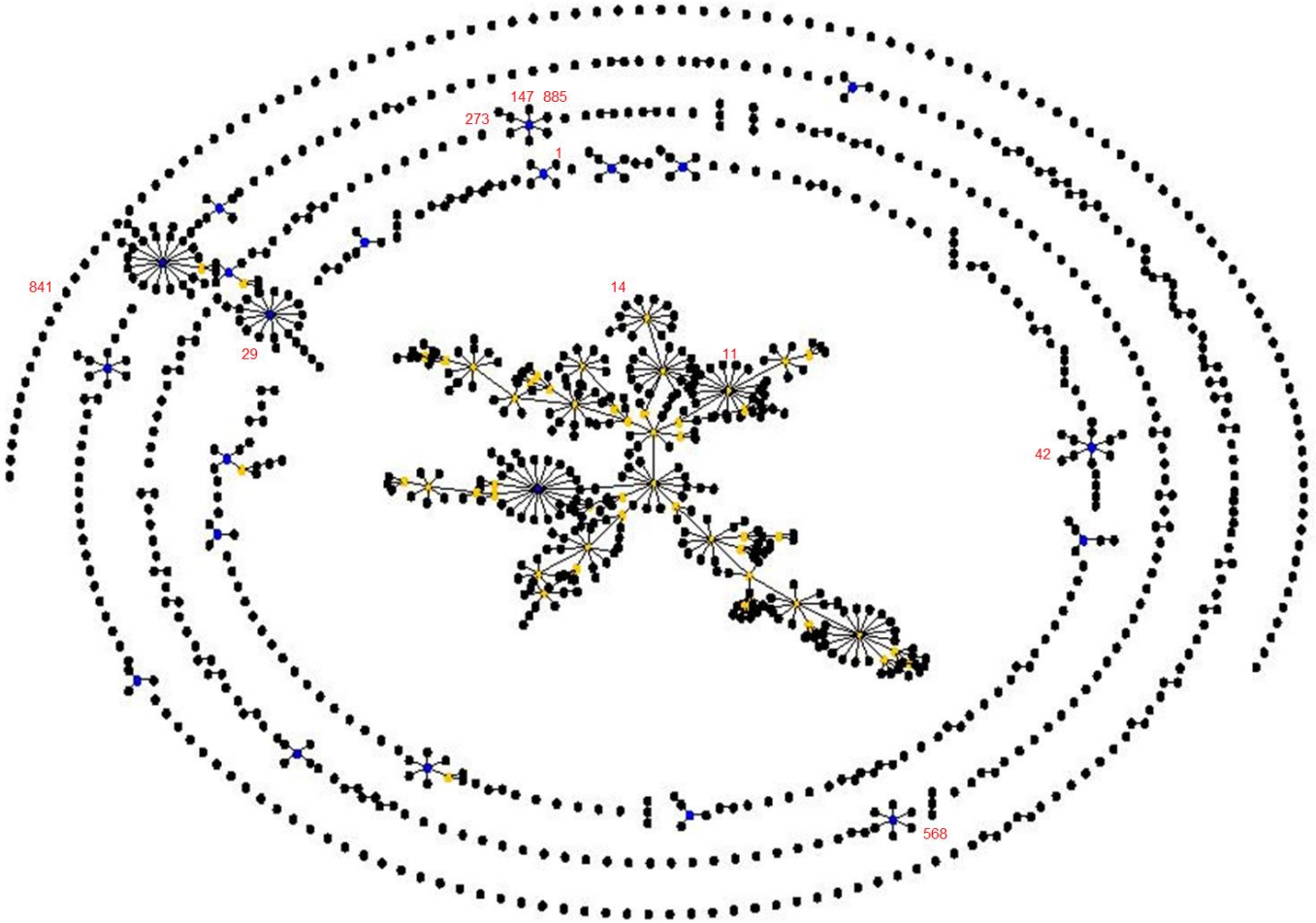


Figure 11 ‘Population Snapshot’ showing the clusters of linked and unlinked STs in the whole *K. pneumoniae* MLST database (19 January 2013). ST labels have been removed except for those of isolates from this study (in red). The predicted primary founder of a clonal complex is coloured blue and the subgroup founder is coloured yellow.

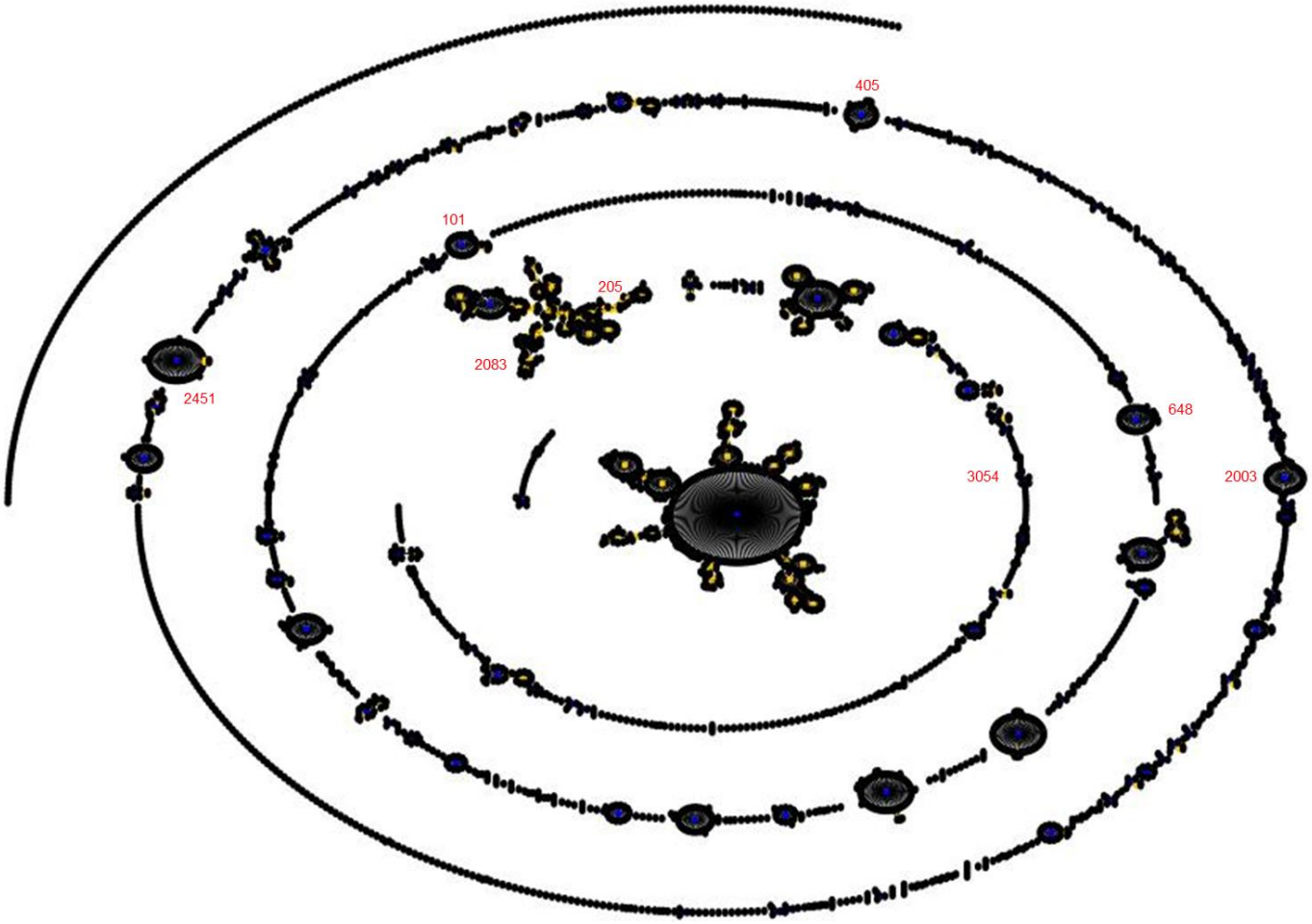
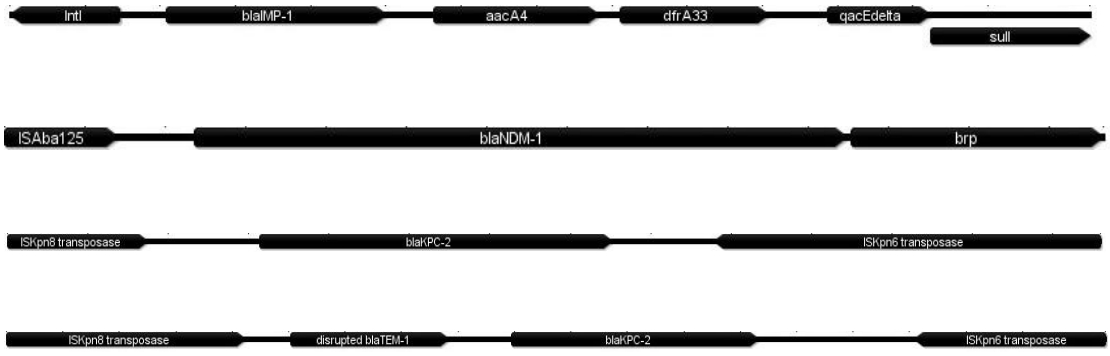


Figure 12 ‘Population Snapshot’ showing the clusters of linked and unlinked STs in the whole *E. coli* MLST database (19 January 2013). ST labels have been removed except for those of isolates from this study (in red). The predicted primary founder of a clonal complex is coloured blue and the subgroup founder is coloured yellow.

Sequencing of flanking regions of carbapenemase genes

For isolates with $bla_{\text{IMP-1}}$, we were only able to obtain integron sequences for DU37041 and DB44834. Both were identical with $bla_{\text{IMP-1}}$ followed by an aminoglycoside 6'-N-acetyltransferase gene *aac(6')-Ib (aacA4)* and a dihydrofolate reductase gene *dfrA33* (GenBank accession number KC200566). $bla_{\text{NDM-1}}$ was always flanked by IS*Aba125* and a bleomycin resistance protein (brp) gene. The flanking regions of $bla_{\text{KPC-2}}$ for DB2244 differed from the other $bla_{\text{KPC-2}}$-positive isolates by lacking a truncated $bla_{\text{TEM-1}}$ gene. We were unable to obtain flanking sequences for $bla_{\text{OXA-48}}$ and $bla_{\text{OXA-181}}$.

Figure 13 Flanking regions of carbapenemase genes in this study.



Hybridization of probes to Southern blots of S1 nuclease digested plasmids carrying carbapenemase genes

The hybridization results are given below. The sizes of the plasmids carrying carbapenemase genes are given in Table 18.

Figure 14 Hybridization of *bla*$_{IMP}$ probe to S1 nuclease-digested DNA of IMP-producing isolates (linearized plasmid indicated by arrows). Each band of the lambda ladder represents an increment of 48.5 kb over the previous one below it.

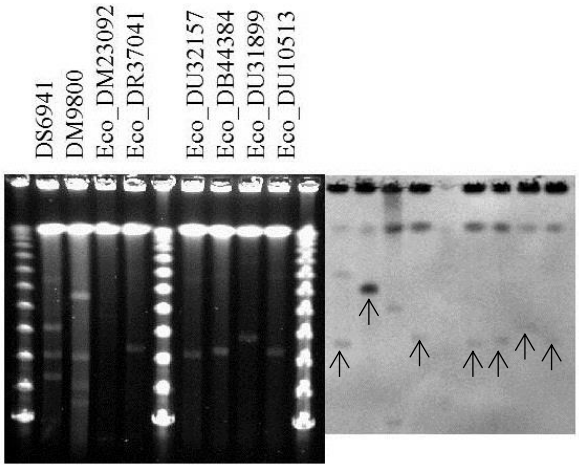


Figure 15 Hybridization of *bla*$_{NDM}$ probe to S1 nuclease-digested DNA of NDM-producing isolates (linearized plasmid indicated by arrows). Each band of the lambda ladder represents an increment of 48.5 kb over the previous one below it (DM10361 is not part of this study).

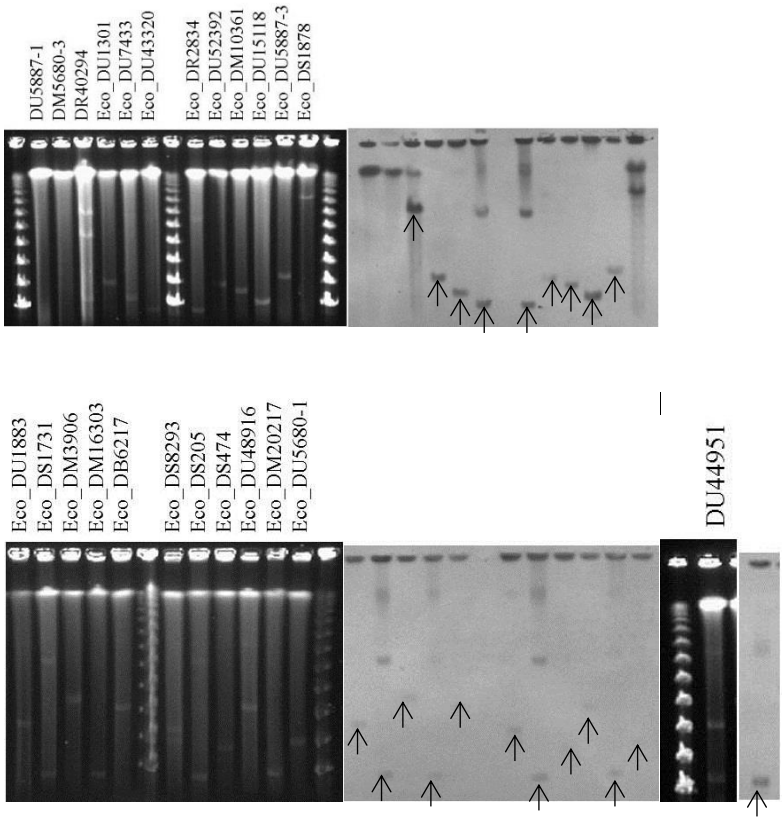


Figure 16 Hybridization of *bla*$_{KPC}$ probe to S1 nuclease-digested DNA of KPC-producing isolates (linearized plasmid indicated by arrows). Each band of the lambda ladder represents an increment of 48.5 kb over the previous one below it.

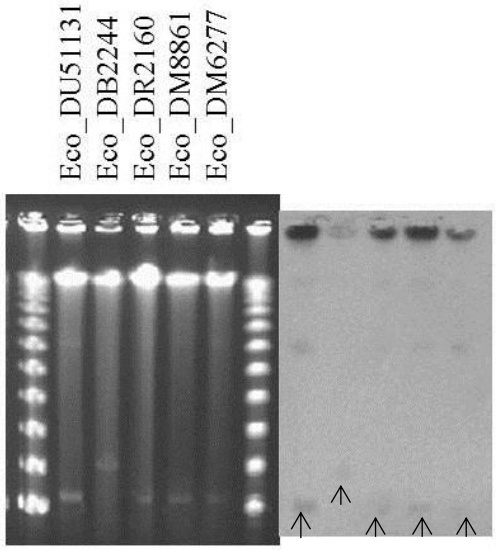


Figure 17 Hybridization of *bla*$_{\text{OXA-181}}$ probe to S1 nuclease-digested DNA of OXA-181-producing isolates (linearized plasmid indicated by arrows). Each band of the lambda ladder represents an increment of 48.5 kb over the previous one below it.

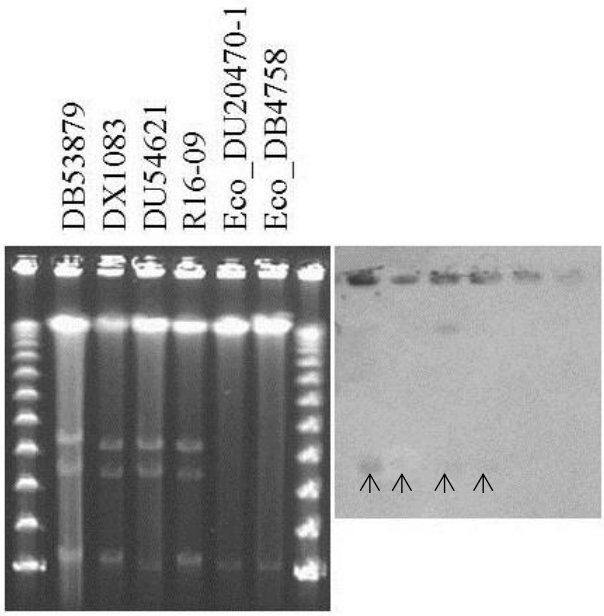
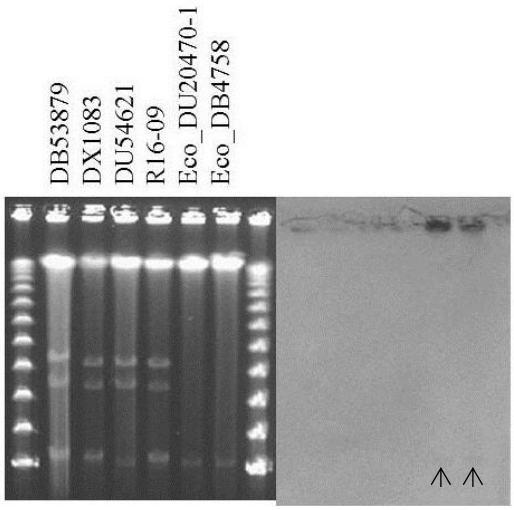


Figure 18 Hybridization of *bla*$_{\text{OXA-48}}$ probe to S1 nuclease-digested DNA of OXA-48-producing isolates (linearized plasmid indicated by arrows). Each band of the lambda ladder represents an increment of 48.5 kb over the previous one below it.



Plasmid fingerprinting and PCR based plasmid replicon typing

The results are summarized in Table 18. The c.a. 150-160 kb plasmids in *K. pneumoniae* DB44384, DR37041, DU32157 and *C. freundii* DU10513 with $bla_{\text{IMP-1}}$ had the same restriction fingerprint and all belonged to plasmid replicon type A/C. The c.a. 40 kb plasmid with $bla_{\text{NDM-1}}$ in *K. pneumoniae* DU43320, DR2834, DS1731 and *E. cloacae* DM16303 had the same restriction fingerprint and was untypable by PCR based plasmid replicon typing. The c.a. 150 kb plasmids with $bla_{\text{NDM-1}}$ in *E. cloacae* DB6217 and *E. coli* DU48916 had the same restriction fingerprint. Both belonged to plasmid replicon type A/C. The transferable c.a. 50 kb plasmid with $bla_{\text{OXA-48}}$ in *K. pneumoniae* DU20470-1 and *E. coli* DB4758 had the same restriction fingerprint and both were untypable by PCR based plasmid replicon typing.

Figure 19 *Hind*III digestions of c.a. 150-160 kb plasmids bearing $bla_{\text{IMP-1}}$. Lane 1 pEco_DR37041, Lane 2 pEco_DB44384, Lane 3 pEco_DU10513, Lane 4 pEco_DU32157.

1 2 3 4

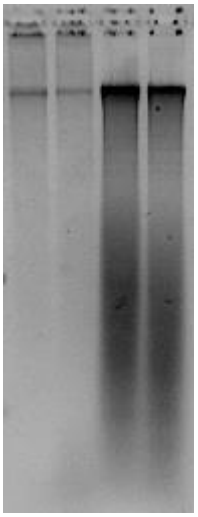


Figure 20 *Pst*I digestions of c.a. 150-160 kb plasmids bearing bla_{IMP-1}. Lane 1 pEco_DR37041, Lane 2 pEco_DB44384, Lane 3 pEco_DU10513, Lane 4 pEco_DU32157.

1 2 3 4

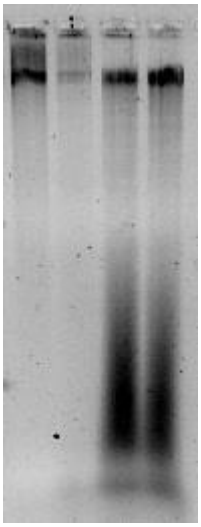


Figure 21 *Hind*III digestions of c.a. 40 kb plasmids bearing bla_{NDM}. Lane 1 pEco_DU43320, Lane 2 pEco_DR2834, Lane 3 pEco_DS1731, Lane 4 pEco_DM16303, Lane 5 pEco_DS205, Lane 6 pEco_DM20217.

1 2 3 4 5 6

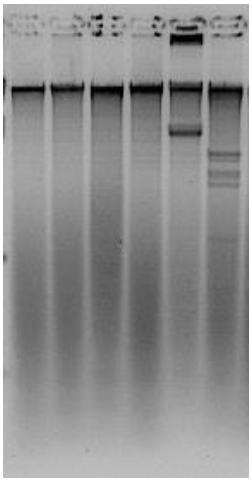


Figure 22 *Pst*I digestions of c.a. 40 kb plasmids bearing bla_{NDM}. Lane 1 pEco_DU43320, Lane 2 pEco_DR2834, Lane 3 pEco_DS1731, Lane 4 pEco_DM16303, Lane 5 pEco_DS205, Lane 6 pEco_DM20217.

1 2 3 4 5 6

Figure 23 *Hind*III digestions of c.a. 60-80 kb plasmids bearing bla_{NDM}. Lane 1 pEco_DU7433, Lane 2 pEco_DU15118, Lane 3 pEco_DS474, Lane 4 pEco_DM10361 (not part of this study), Lane 5 pEco_DU1301, Lane 6 pEco_DU52392, Lane 7 pEco_DU5680-1.

1 2 3 4 5 6 7

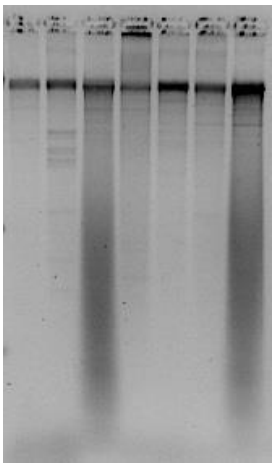


Figure 24 *Pst*I digestions of c.a. 60-80 kb plasmids bearing bla_{NDM}. Lane 1 pEco_DU7433, Lane 2 pEco_DU15118, Lane 3 pEco_DS474, Lane 4 pEco_DM10361 (not part of this study), Lane 5 pEco_DU1301, Lane 6 pEco_DU52392, Lane 7 pEco_DU5680-1.

1 2 3 4 5 6 7

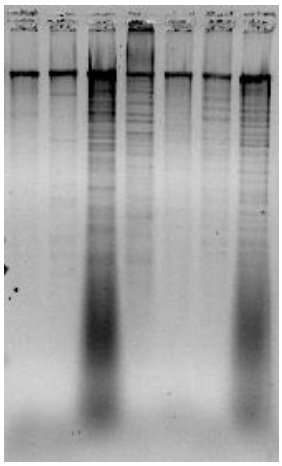


Figure 25 *Hind*III digestions of c.a. 110-150 kb plasmids bearing bla_{NDM}. Lane 1 pEco_DU5887-3, Lane 2 pEco_DU1883, Lane 3 pEco_DS8293, Lane 4 pEco_DB6217, Lane 5 pEco_DU48916.

1 2 3 4 5

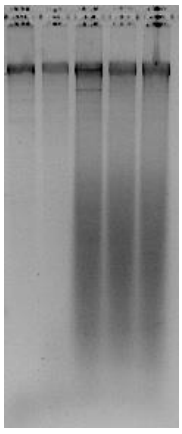


Figure 26 *Pst*I digestions of c.a. 110-150 kb plasmids bearing bla_{NDM}. Lane 1 pEco_DU5887-3, Lane 2 pEco_DU1883, Lane 3 pEco_DS8293, Lane 4 pEco_DB6217, Lane 5 pEco_DU48916.

1 2 3 4 5

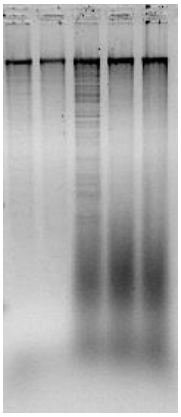


Figure 27 *Hind*III digestions of plasmids bearing bla_{OXA-48} and bla_{KPC-2}. Lane 1 pEco_DU20470-1, Lane 2 pEco_DB4758, Lane 3 pEco_DU51131, Lane 4 pEco_DR2160, Lane 5 pEco_DM8861, Lane 6 pEco_DM6277.

1 2 3 4 5 6

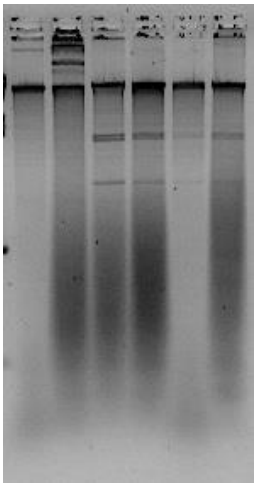
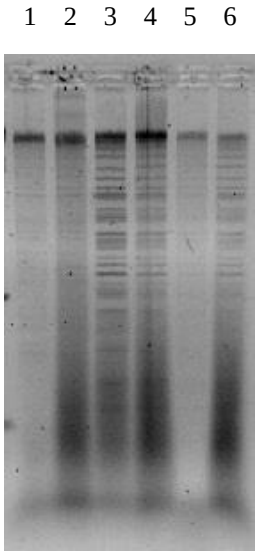


Figure 28 *Pst*I digestions of plasmids bearing $bla_{\text{OXA-48}}$ and $bla_{\text{KPC-2}}$. Lane 1 pEco_DU20470-1, Lane 2 pEco_DB4758, Lane 3 pEco_DU51131, Lane 4 pEco_DR2160, Lane 5 pEco_DM8861, Lane 6 pEco_DM6277.



Discussion

There were 8 isolates with *bla*$_{IMP}$ (Five *K. pneumoniae*, 2 *E. cloacae* and 1 *C. freundii*). Despite having been present in Singapore since 1996, no outbreak clone of *bla*$_{IMP-1}$ isolates has emerged. DS6941 carried *bla*$_{IMP-4}$ which is a variant that has been described in *Enterobacteriaceae* from Australia and China, and is also found in *A. nosocomialis* and *A. pittii* in this region (61, 126, 127). The only integron sequence we were able to obtain was different from *K. pneumoniae* from São Paulo, Brazil that had *bla*$_{IMP-1}$ –carrying integrons with *aac(6')-31 and aadA1*, and Japanese isolates from Saitama that did not possess *aac(6')-Ib* (128, 129). Though still relatively rare, the geographical distribution of *Enterobacteriaceae* carrying *bla*$_{IMP-1}$ seems to have expanded recently (130, 131).

There were 22 isolates with *bla*$_{NDM}$ (Eleven *K. pneumoniae*, 6. *E. coli*, 4 *E. cloacae*, and 1 *C. sedlakii*). Some of the *K. pneumonia*e with *bla*$_{NDM-1}$ in our study belonged to ST11, ST14 and ST147 which were also the most common MLST types among *bla*$_{NDM-1}$-positive *K. pneumoniae* in India and the United Kingdom (132). Though the *E. coli* with *bla*$_{NDM-1}$ from our study were diverse, one isolate belonged to ST101 and another to ST405 which were among the most common STs associated with *bla*$_{NDM-1}$-positive *E. coli* in Pakistan and the United Kingdom (133). NDM-7 found in *E. coli* DM20217 is a newly described variant that has been found in *E. coli* from Myanmar (GenBank accession number AFS33103.1) and Canada (GenBank accession number

AFQ31613.1) and differs from NDM-1 by 2 amino acids at positions 130 (Asp to Asn) and 154 (Met to Leu).

The c.a. 40 kb plasmid from DU43320 that was untypable by PCR based plasmid replicon typing has been completely sequenced and was found to be identical to that in DU44951 which was not successfully conjugated or transformed (134). These plasmids were structurally similar to an IncN2 plasmid previously described in an *E. coli* from Bangladesh (135).

E. cloacae DM5887-3, *Citrobacter sediakii* DM5680-1, together with a *P. aeruginosa* and *P. putida*, were isolated from an unfortunate lady who had a road traffic accident in India, where she was initially hospitalized before being transported back to Singapore. After we completed this study, a *K. pneumoniae* (DM10361 see Figure 15) with $bla_{\text{NDM-1}}$ was also subsequently isolated from this patient. All three *Enterobacteriaceae* carried $bla_{\text{NDM-1}}$ on plasmids of different sizes. The two Pseudomonads were also positive for $bla_{\text{NDM-1}}$ but the gene did not appear to be plasmid-borne. This case illustrates well the ability of $bla_{\text{NDM-1}}$ to infiltrate into different bacterial species.

There were 4 *K. pneumoniae* with $bla_{\text{OXA-181}}$ and they were all clonal by PFGE. In our isolates, $bla_{\text{OXA-181}}$ gene was localized to a large plasmid that was not able to conjugate or transform. By contrast a small c.a. 7 kb plasmid bearing $bla_{\text{OXA-181}}$ that was also not transferable by conjugation has been

described from *K. pneumoniae* isolated in Oman and the United Kingdom, associated with travel to India (53, 136).

Both isolates with $bla_{\text{OXA-48}}$ were isolated from Singaporeans. This is the first time this carbapenemase gene has been detected from isolates in Southeast Asia. We are uncertain how $bla_{\text{OXA-48}}$ entered Singapore as we were unable to obtain a history of recent travel from the patients and neither isolate belonged to MLST clones that have been associated with outbreaks in other countries. The c.a. 50 kb plasmid with $bla_{\text{OXA-48}}$ in *K. pneumoniae* DU20470-1 and *E. coli* DB4758 that was untypable by PCR based plasmid replicon typing may be distinct from the c.a. 62 kb IncL/M plasmid that has disseminated in Europe, the Middle East, and North Africa (137). Plasmids of this size bearing $bla_{\text{OXA-48}}$ have also been described in the United Kingdom (136).

$bla_{\text{OXA-181}}$ and $bla_{\text{OXA-48}}$ have been associated with IS*EcpI* and IS*1999* respectively (53, 138). Our inability to sequence the flanking regions to these genes in our isolates further suggests that the origin of these isolates may be different from those already described.

There were 3 *K. pneumoniae*, 1 *E. coli*, and 1 *E. cloacae* with $bla_{\text{KPC-2}}$. *K. pneumoniae* with $bla_{\text{KPC-2}}$ have previously described in Singapore by Balm *et al* (139). Those isolates were clonal, belonged to ST11, and the sequences flanking $bla_{\text{KPC-2}}$ were identical to that of pKP048 from China. In our study,

all 3 *K. pneumoniae* with bla_{KPC-2} had distinct PFGE patterns. One isolate, DB2244 had an identical PFGE pattern to those in the previous study (personal communication Dr Jeanette Teo). The flanking regions of bla_{KPC-2} for DB2244 were also similar to that reported in *K. pneumoniae* KP048 plasmid pK048. In the remaining isolates, the flanking regions of bla_{KPC-2} included a truncated bla_{TEM} gene upstream, and were similar to that reported as Variant 1 by Shen *et al* (121). In these isolates bla_{KPC-2} was carried on an identical c.a. 50 kb plasmid that was untypable by plasmid replicon typing.

In 1996, Singapore became one of the first countries to isolate a plasmid-mediated carbapenemase-producing *Enterobacteriaceae* (CRE). However, the expected increase in carbapenem resistance did not occur until 2010. The first carbapenemase gene to be described locally, bla_{IMP-1}, failed to become a significant problem despite the fact that it is established in *P. aeruginosa* in Singapore (59). The present diversity of carbapenemase genes reflects Singapore's geographical location at an international crossroads in proximity to India, China, Australia, and Japan as well as its role as a hub for international travel.

The recent increase in *Enterobacteriaceae* with carbapenemase genes coincides with their discovery overseas. This is clearly linked to importation from overseas in the cases of bla_{NDM-1}, and $bla_{OXA-181}$, though recent isolates have not been obviously imported. The point when bla_{IMP}, bla_{OXA-48} and bla_{KPC-2} entered Singapore is not obvious. The association with carbapenem

usage is also unclear. Carbapenem prescriptions have only increased slightly in Singapore overall. However, there has been increased use of ertapenem and meropenem at the expense of imipenem (140). The findings of this study underscore the need for systematic molecular surveillance to fully understand the global spread of carbapenemase genes in *Enterobacteriaceae*.

Chapter 5 Discussion

A brief reprise of themes introduced in the opening chapter

Carbapenemase-producing (and by implication multidrug-resistant) Gram-negative bacilli are becoming an increasing problem worldwide. Initially the problem was largely confined to *P. aeruginosa* and *A. baumannii*, pathogens already possessing a predisposition to develop resistance to any antimicrobial agent. Infections with these pathogens were usually limited to nosocomial settings like the intensive care unit where carbapenem use was heavy and the patients had compromised immune systems. However in the last decade there has been a dramatic increase in carbapenemases among the hitherto unaffected *Enterobactericeae* (141). Because these bacteria are involved in a wider range of infectious diseases, the scope of infections that are difficult to treat with available antimicrobials has increased. Furthermore, CRE have been found in the environment, raising the spectre of untreatable infections presenting from the community (37).

The problem is being compounded by the lack of readily available alternatives. Much attention in the recent past has been focussed on antimicrobial resistance in Gram-positive pathogens like penicillin-resistant *Streptococcus pneumoniae*, methicillin-resistant *Staphylococcus aureus*, and glycopeptide-resistant *Enterococci*. This has led to a flurry of drug development resulting in new antimicrobials like daptomycin, linezolid,

telavancin, ceftaroline, and quinoprostin-dalfopristin (142). Drug development for multidrug-resistant Gram-negative bacilli has been slower and much less productive. The first really new drug to reach the formulary was tigecycline. However susceptibility may be variable and its efficacy in severe infections has been questioned (143). Desperate clinicians have had to resort to combination therapy, often including older antimicrobials like fosfomycin and the polymixins, guided by little or no evidence (144).

In the introductory chapter we introduced the theme of how each time a new antimicrobial is introduced, the bacteria have been able to respond with an appropriate resistance mechanism. In fact carbapenem resistance may arise due to a variety of mechanisms. Carbapenem resistance in *P. aeruginosa* is often the result of multi-drug efflux in combination with impermeability in the presence of continued expression of the chromosomal cephalosporinase (27). These potential mechanisms of carbepenem resistance are intrinsic to every isolate of *P. aeruginosa*. Of the 123 isolates of imipenem-resistant *P. aeruginosa* isolated in 2008, less than 10% were carbapenemase positive implying that intrinsic mechanisms accounted for the majority of carbapenem resistance. Similarly, a combination of extended-spectrum or pAmpC β-lactamase hyper-production and diminished porin expression may lead to carbapenem resistance in *Enterobacteriaceae* (145). These mechanisms were likely to be responsible for many of the initial isolates of CRE in Singapore. Between 2004 and 2006, 40% of the carbepenem-resistant *K. pneumoniae* possessed the gene for the pAmpC β-lactamase DHA-1 (69). Of six carbapenem-resistant *E. coli* isolated between 2006 and 2008, all possessed

either the gene for the pAmpC β-lactamase CMY-2, the gene for a CTX-M-Group 1 ESBL, or both (unpublished data). Nevertheless, the acquired carbapenemases represent a very different and specific response to carbapenems as neither the intrinsic mechanisms of carbapenem resistance in *P. aeruginosa* nor the pAmpC and ESBLs in *Enterobacteriaceae* are transferable mechanisms of carbapenem resistance. In the first chapter, a variety of acquired carbapenemases was introduced. The data presented in this thesis shows that all major acquired carbapenemase genes can be found in Singapore (see Table 19). This is a remarkable variety of resistance mechanisms for a small country. By comparison, South Korea with a population 10 times larger but more homogenous than that of Singapore, has reported 6 acquired carbapenemases (VIM-2, IMP-6, OXA-23, NDM-1, KPC, SIM-1) among Gram-negative bacilli (146).

Table 19 Acquired carbapenemase genes found in Singapore.

Carbapenemase gene	Species	Reference
$bla_{\text{IMP-1}}$	*P. aeruginosa*	(59)
	P. putida	(60)
	P. fluorescens	(60)
	K. pneumoniae	(58)
	E. cloacae	(147)
	Citrobacter freundii	This study
$bla_{\text{IMP-4}}$	*A. nosocomialis*	(62)
	A. pittii	(62)

	K. pneumoniae	This study
bla$_{\text{IMP-26}}$	*P. aeruginosa*	(148)
bla$_{\text{VIM-2}}$	*P. aeruginosa*	(148)
bla$_{\text{VIM-6}}$	*P. putida*	(60)
bla$_{\text{NDM-1}}$	*K. pneumoniae*	(149)
	E. coli	(147)
	E. cloacae	(147)
	Proteus mirabilis	(147)
	P. aeruginosa	(147)
	P. putida	This study
	C. sediakii	This study
bla$_{\text{NDM-7}}$	*E. coli*	This study
bla$_{\text{KPC-2}}$	*K. pneumoniae*	(139)
	E. coli	This study
	E. cloacae	This study
bla$_{\text{OXA-48}}$	*K. pneumoniae*	This study
	E. coli	This study
bla$_{\text{OXA-181}}$	*K. pneumoniae*	(150)
bla$_{\text{OXA-23}}$	*A. baumannii*	(61)
	A. nosocomialis	(151)
bla$_{\text{OXA-27}}$	*A. baumannii*	(152)
bla$_{\text{OXA-58}}$	*A. baumannii*	(151)
	A. nosocomialis	(62)
	A. pittii	(62)
bla$_{\text{OXA-96}}$	*A. baumannii*	(61)

We began this study by looking at the two most common carbapenem-resistant pathogens in Singapore, *P. aeruginosa* and Acb. During the course of our investigations, we were also able to witness at first hand the emergence of a new threat of carbepenem-resistance in the *Enterobactericeae* and this was incorporated into the study.

In general, two layers of molecular typing were employed. The first layer was a more discriminatory fingerprinting method used to determine the presence of clones within the local population of isolates. In the case of *P. aeruginosa* and the *Enterobacteriaceae* we used PFGE. For *A. baumannii* we used RAPD-PCR rather than PFGE because it is a rapid, economical, standardized fingerprinting method for *A. baumannii*. There is no equivalent standardized PCR method for fingerprinting *P. aeruginosa* and *Enterobacteriaceae*. PFGE is more expensive, time-consuming and tedious to perform compared with PCR methods. The advantages of PFGE are better reproducibility and perhaps better discrimination. In retrospect, it is possible we would have obtained better data using PFGE instead of RAPD-PCR for fingerprinting *A. baumannii*. Both these typing methods allow good discrimination between isolates for the investigation of outbreaks but do not allow for inter-laboratory comparisons.

From the fingerprinting data, representative isolates were selected for a second layer of typing. This was primarily MLST. Whilst poor at defining outbreaks, this method being sequence-based is objective, transferable and allows inter-

laboratory comparisons. For *A. baumannii* AFLP™ was also performed because some of the initial designation of International clones was done by AFLP™, which preceded the use of MLST for categorizing this species.

The characterization of CRE was more detailed. This included trying to sequence the flanking regions of carbapenemase genes and characterization of the plasmids. This extra effort was justified because whereas carbapenemase-producing *P. aeruginosa* and Acb have been around for longer and their genetic frameworks are relatively established, CRE are a very new worldwide phenomenon and much less is known.

Major findings regarding acquired carbapenemases in *P. aeruginosa*

The percentage of carbapenemase-producers among carbapenem-resistant *P. aeruginosa* in 2008 had not increased compared to an earlier study carried out in 2000 (59). This suggests that the situation in *P. aeruginosa* has stabilized, though a caveat is that the sample size is rather small. In the 2000 study, there were two clones. One clone had $bla_{\text{IMP-1}}$ that had a nucleotide sequence identical to the original first reported from Japan (33). The other clone had $bla_{\text{IMP-1v}}$, a sequence variant that differed from the original by four silent mutations. This particular sequence variant has so far only been described in Singapore to date. Neither clone had emerged dominant in 2008 though both variants of $bla_{\text{IMP-1}}$ were still found. Instead the 2008 isolates were quite heterogenous with $bla_{\text{VIM-2}}$ and $bla_{\text{VIM-6}}$ emerging in *P. aeruginosa* for the first

time in Singapore. Two related isolates in 2008 had *bla*$_{IMP-7}$, but were distinct from the *bla*$_{IMP-7}$-positive isolate from 2000. A novel MBL *bla*$_{IMP-26}$ that is related to *bla*$_{IMP-4}$ was also described. The two clones from 2000 belonged to ST964 (CC654) and ST233 (CC233). Some isolates in 2008 belonged to these clonal complexes but had different STs and slightly different PFGE patterns. The distribution of *bla*$_{IMP-1}$ variants between the two clonal complexes remained consistent from 2000 to 2008. Only isolates from 2008 belonged to CC235 and they contained a mix of MBL genes.

Major findings regarding acquired carbapenemases in Acb

This study was in two parts. The first was a cross-sectional snap-shot of Acb isolated in public hospitals over 1 month in 2006. This showed that *A. baumanni* was the most common (78.7%) followed by *A. nosocomialis* (11.9%), and *A. pittii* (9.3%). Carbapenem resistance was highest in *A. baumannii* (72.4%) but was also significant in *A. pittii* (27.8%) and *A. nososcomialis* (34.8%). Most carbapenem-resistant *A. baumannii* and *A. nosocomialis* possessed the *bla*$_{OXA-23-like}$ gene which was usually preceded by IS*Aba1*. Most carbapenem-resistant *A. pittii* had the *bla*$_{OXA-58-like}$ gene which was sometimes preceded by IS*Aba3*. One *A. baumannii* and one *A, nosocomialis* also had *bla*$_{IMP-like}$ genes.

The second part of the study was a typing exercise on a limited number of isolates from 2006, and from the archive collection of Hospital S. The main

outbreak clone of *A. baumannii* from Hospital S in 1996 that contained $bla_{\text{OXA-66}}$ belonged to ST25. Another outbreak clone from 1996 that contained $bla_{\text{OXA-88}}$ belonged to novel ST111. Both had AFLP™ profiles that were unrelated to European (International) clones I-III. The main outbreak clone from Hospital S in 2001 contained $bla_{\text{OXA-64}}$, and belonged to European clone II and ST2. Related isolates could still be found in this hospital in 2006. Another outbreak clone from Hospital S in 2001 containing $bla_{\text{OXA-69}}$ belonged to European clone I and ST1. A number of isolates collected from other hospitals in 2006 also belonged to European clones I and II.

Major Findings regarding acquired carbapenemases in *Enterobacteriaceae*

This study is the first to document the appearance of $bla_{\text{IMP-4}}$, $bla_{\text{NDM-1}}$, $bla_{\text{NDM-7}}$, $bla_{\text{OXA-181}}$, and $bla_{\text{OXA-48}}$ in Singapore. Acquired carbapenemases were found mainly in *K. pneumoniae* followed by *E. coli*. Although $bla_{\text{IMP-1}}$ was the first carbapenemase gene to be described in *Enterobacteriaceae* in Singapore it has not become established in large numbers. Most CRE were isolated from 2010 onwards. The largest group were *Enterobacteriaceae* that carried $bla_{\text{NDM-1}}$.The only large outbreak clone was $bla_{\text{OXA-181}}$-positive *K. pneumoniae* that were related by PFGE to some isolates with $bla_{\text{NDM-1}}$. These isolates belonged to ST14. A number of *K. pneumoniae* with $bla_{\text{NDM-1}}$ also belonged to one of two small PFGE clusters corresponding to ST273 or ST147. Even though *K. pneumoniae* with $bla_{\text{KPC-2}}$ were not clonal by PFGE, they all belonged to ST11. Carbapenemase-producing *E. coli* and *E. cloacae* were not related by PFGE. A c.a. 150-160 kb plasmid bearing $bla_{\text{IMP-1}}$ and

belonging to plasmid replicon type A/C was common to three *K. pneumoniae* and one *C. freundii* isolate. A c.a. 40 kb untypable plasmid bearing $bla_{\text{NDM-1}}$ was common to three *K. pneumoniae* and one *E. cloacae* isolate. A c.a. 150 kb type A/C plasmid with $bla_{\text{NDM-1}}$ was found in an *E. cloacae* and *E. coli*. $bla_{\text{OXA-181}}$ was found on a c.a. 150 kb plasmid that was not transferable. $bla_{\text{OXA-48}}$ was found on identical untypable plasmids in *E. coli* and *K. pneumoniae*. The flanking regions of $bla_{\text{KPC-2}}$ resembled those of strains in China. These sequences were found on identical c.a. 50 kb plasmids that were untypable.

The role of international outbreak clonal complexes

All three studies show the recent influence of international outbreak clonal complexes. Though the individual isolates may be unrelated by discriminatory fingerprinting methods, these clonal complexes represent evolutionary lineages that have successfully disseminated in other countries.

Internationally, clonal complexes CC111 and CC235 dominate among MBL-producing *P. aeruginosa* (153), though ST621 has also been associated with international dissemination of $bla_{\text{IMP-13}}$ (154). ST235 was the most common sequence type among IMP-producing *P. aeruginosa* in Japan (155) and Korea (156), though it was also found among European isolates producing VIM-type MBLs (157). On the other hand, CC111 was largely confined to Europe. VIM-2-producing *P. aeruginosa* causing an outbreak in an Italian Hospital belonged

to ST111 (158). Nationwide surveillance of Dutch hospitals in 2010-2011 found that the majority of VIM-2-producing *P. aeruginosa* were ST111 (159). The outbreak isolates from Singapore in 2000 did not belong to either of these clonal complexes. Furthermore, these sequence types have not persisted locally nor have they been documented to have caused outbreaks overseas. ST235 isolates only appeared in Singapore from 2008. These isolates carried a variety of MBL genes and no clone had emerged at the time of the study.

Like the *P. aeruginosa*, the original carbapenemase-producing *A. baumannii* in Singapore did not belong to any of the European (International) clones I-III. These clones initially defined by AFLP™ correspond to MLST clonal complexes CC1-3 in the Institute Pasteur Scheme. CC1 and CC2 are responsible for much of the spread of carbapenemase-producing *A. baumanii* globally (160). This is possibly because they are very successful vehicles for the carriage of $bla_{\text{OXA-23}}$ (93). However in Italy, carbapenem resistance in *A. baumannii* was mostly due to $bla_{\text{OXA-58}}$ rather than $bla_{\text{OXA-23}}$. The most widespread clone $bla_{\text{OXA-58}}$-positive ST4 (CC92) found in 5 Italian hospitals was related to International Clone II (161). Isolates belonging to these clonal complexes only started to appear in Singapore from 2001. There were no representatives from CC3 which seems to be localized to Europe (160).

Unlike *P. aeruginosa* and *A. baumannii*, the appearance of carbapenemase genes in *Enterobacteriaceae* in large numbers is fairly recent. As our study shows, the ability of some of the plasmids bearing $bla_{\text{IMP-1}}$, $bla_{\text{NDM-1}}$, $bla_{\text{KPC-2}}$

and *bla*$_{OXA-48}$ to readily transfer between strains means that resistance continues to spread without the need for epidemic clones of bacteria. Nevertheless, there are particular clones of *Enterobacteriaceae* that are notable for spreading antimicrobial resistance. *K. pneumoniae* ST258 is associated with the spread of *bla*$_{KPC-2}$ in North America, Israel and Europe (162). On the other hand, the spread of *bla*$_{KPC-2}$ in China, Taiwan and Singapore is largely due to ST11 (139, 163, 164). ST11, ST14 and ST258 together form one large clonal complex (CC292) responsible for the global spread of diverse resistance elements including extended-spectrum β-lactamases, *bla*$_{NDM-1}$, and *bla*$_{VIM-1}$ (165). Despite being the first acquired carbapenemase gene to be found in *Enterobacteriaceae* in Singapore, *bla*$_{IMP-1}$ has not become established. While the exact reason for this remains unknown, it could have something to do with the fact that plasmids bearing this gene have been relatively unsuccessful at entering strains belonging to international outbreak clonal complexes.

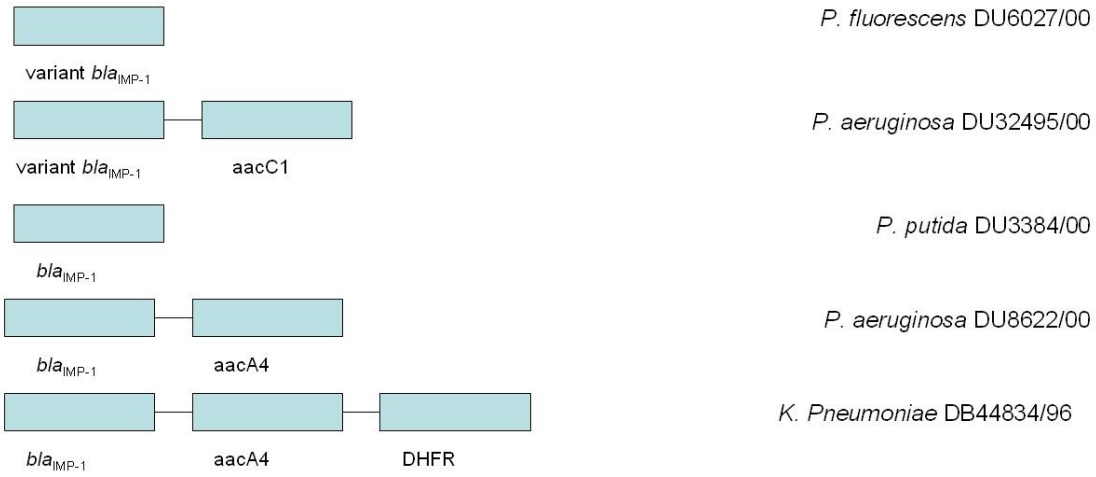
There was no outbreak clone of *E. coli* in our study though some isolates belonged to international clones. *E. coli* ST101, ST648 and ST405 have been associated with spread of *bla*$_{NDM-1}$ in England and Pakistan (133). Individual cases of *bla*$_{NDM-1}$-positive *E. coli* ST101 and ST405 have also been imported to Australia (166), Canada (167), and Germany (168), highlighting their epidemic potential.

The results for *A. baumannii* and *P. aeruginosa* illustrate the point that bacteria may undergo significant shifts in the population which will not be revealed without the application of molecular typing methods like MLST. The recent increase of international clones in these two species taken together with the MLST results for *Enterobacteriaceae* suggest a greater role for overseas travel in the spread of carbapenemase genes into Singapore since the beginning of the 21st century.

Diversity of acquired carbapenemases found in Singapore

The diversity of acquired carbapenemases found in Singapore seems to reflect the geo-economic contacts with other countries though there are some inconsistencies. The situation in Singapore is very similar to that of Japan where $bla_{\text{IMP-1}}$ may be found in *P. aeruginosa* and *K. pneumoniae*. However in Japan, $bla_{\text{IMP-1}}$ is regularly found in *S. marcescens* (169), whereas it is has never been described in this species in Singapore. Similarly $bla_{\text{IMP-1}}$ in Korea has spread between *Pseudomonas* spp. and *Acinetobacter* spp. (170) whereas this gene has not really penetrated into *Acinetobacter* spp. in Singapore despite the fact that this species is often found on the same patients who are likely to harbour carbapenemase-producing *P. aeruginosa*. Comparing the few integrons we have been able to sequence, there seems to be an increase in the number of gene cassettes with resistance determinants going from environmental fluorescent *Pseudomonas* spp. to *P. aeruginosa* and *K. pneumoniae*. The numbers are too small but this would be in keeping with a reservoir of $bla_{\text{IMP-1}}$ in environmental *Pseudomonas* spp.

Figure 29 Gene cassettes inserted into integrons which contain *bla*$_{IMP-1}$.



IMP-4 has 95.6% homology with IMP-1. *bla*$_{IMP-4}$ was first described in *Citrobacter youngae* in China (127) and non-*baumannii* Acb in Hong Kong (75). It has subsequently appeared sporadically in a diversity of Gram-negative bacilli in Australia (126) and *Acinetobacter* spp. and *P. aeruginosa* in Malaysia (171, 172). *bla*$_{IMP-4}$-positive *K. pneumoniae* were recently isolated from three patients with no travel history in the United States and appears to be spreading in China (173-175). Unusually, the American and Chinese isolates were from children. *bla*$_{IMP-4}$ was first found in Singapore in *Acinetobacter* spp. isolated in 1996 (61). Three of the four strains were isolated from haematology patients from Indonesia. Although we have since also found *bla*$_{IMP-4}$ in *K. pneumoniae*, it is unlikely that inter-species transfer has occurred. The flanking sequences of *bla*$_{IMP-4}$ in *Acinetobacter* spp. showed a class I integron that contained in order *bla*$_{IMP-4}$, *qacG*, *aacA4* and *catB3* (61). This order of genes was identical to that of *Acinetobacter* spp. from Hong Kong and *K. pneumoniae* from Australia. However, for the Singapore *K. pneumoniae* isolate, we were only able to obtain the sequence

upstream of *bla*$_{IMP-4}$ which showed the presence of a class I integron. We were unable to obtain the downstream flanking sequence despite repeated attempts suggesting some disruption of the 3' non-variable region of the integron.

While *bla*$_{IMP-1}$ seems likely to have originated from Japan and *bla*$_{IMP-4}$ from China, the distribution of *bla*$_{IMP-7}$ is more difficult to explain. IMP-7 shares >86% amino acid identity with other IMP enzymes. It was first reported in Canada followed by Malaysia and seems established in these 2 countries (176-179). The Canadian isolates had the integron structure *orf1*, *aacC4*, *bla*$_{IMP-7}$, *aacC1*. In 2004, Singapore became the third country to report *bla*$_{IMP-7}$-positive *P. aeruginosa* (59). The 3 isolates described so far belong to different sequence types ST744, ST745 and ST235 (though ST745 is a single locus variant of ST235) (148). The ST744 isolate from 2000 had the integron structure *bla*$_{IMP-7}$, *aacC1* (GenBank accession number AY625685.1) whereas the ST745 isolate from 2008 had the integron structure *bla*$_{IMP-7}$, *aacA4*, *aadA2* (unpublished data). Japan first reported a case of *bla*$_{IMP-7}$ in 2007. The patient had been in hospital in Singapore prior to returning to Japan (79). However a subsequent report from another part of Japan with five isolates suggests that this gene may have been in the country before. Two different integron structures were described, one with *bla*$_{IMP-7}$, *aadA6*, *orfD* and the other *bla*$_{IMP-7}$, *aacC4*, *aadA2* (180). Surprisingly *bla*$_{IMP-7}$ has surfaced in Eastern Europe. The majority of MBL-producing *P. aeruginosa* in Slovakia in 2006 were positive for *bla*$_{IMP-7}$ (181). Subsequently, a clone of ST357 *P. aeruginosa* has spread in Poland and the Czech Republic with an integron structure comprising of *aac(6')-Ib (aacA4)*, *bla*$_{IMP-7}$, *aac(3)-I (aacC1)* (182). It is worth

noting that to date, unlike some of the other *bla*$_{IMP}$ genes, all *bla*$_{IMP-7}$ have only been reported in *P. aeruginosa*. The diversity of sequence types and integron structures suggests that the American, European and Asian clones are distinct.

VIM-6 was first described in two *P. putida* isolated in Singapore in 2000 (60). The amino acid sequence is 99.2% identical to the closely related VIM-2 and VIM-3. The gene was found on a class I integron with the structure *bla*$_{VIM-6}$, *bla*$_{OXA-10}$, *aacA4*, *orf*, *aadA1* (183). In this study we isolated a single *P. aeruginosa* belonging to ST746 with *bla*$_{VIM-6}$ in 2008. The integron structure was *bla*$_{VIM-6}$, *aacA7*, *aadB* (unpublished). Since *bla*$_{VIM-6}$ had not been reported in any other country in the period from 2000 to 2008, it would have been easy to conclude that this gene originated from Singapore. One of the problems with trying to form conclusions based on molecular epidemiology studies is the issue of investigator bias. The distribution of antimicrobial resistance determinants around the world is heavily correlated with the presence of individual investigators in these countries with a research interest in this field. Conversely, there are enormous gaps in knowledge about countries where such research has not been carried out. In 2010, Castanheira *et al* reported the widespread distribution of *bla*$_{VIM-6}$ in MBL-producing *P. aeruginosa* in India collected in 2006 (184). They subsequently also found *bla*$_{VIM-6}$ in *P. aeruginosa* from Indonesia, Korea, and the Philippines (185). Two types of integron structures were described. A 5 kb integron identical to that first reported in *P. putida* from Singapore was found in isolates from Korea, Indonesia and India. A smaller 3.9 kb integron with *bla*$_{VIM-6}$, *bla*$_{OXA-10}$, and *aacA4* was found in isolates from the Philippines and India.

bla$_{NDM-1}$ and *bla*$_{OXA-181}$ were very clearly imported to Singapore from the Indian subcontinent as the entry of these genes into Singapore was temporally linked to the global expansion of their range and we were able to clearly identify the initial imported cases (149, 150). The molecular epidemiology of *K. pneumoniae* with *bla*$_{KPC-2}$ suggests China (ST11) as the source rather than Europe or the United States (ST258). However, in this case we were unable to ascertain how or when this gene entered Singapore. The appearance of *bla*$_{OXA-48}$ was not anticipated as Singapore does not have such close links to Turkey or North Africa where this resistance determinant is usually found. The molecular epidemiology of our isolates (*K. pneumoniae* ST29, plasmid untypable) also does not fit with the outbreak stains from these countries (ST395, IncL/M) (137). It is possible that this resistance determinant was imported from an as yet unrecognized source country.

As we try and draw conclusions about how acquired carbapenemase genes have entered Singapore, it is worth noting that while some genes have been successfully imported, others have not. *bla*$_{VIM-1}$ is a major cause of carbapenem resistance in the European Mediterranean (35). Its absence here can be explained by the geo-political disconnection with Singapore. It is less easy to explain the paucity of *bla*$_{VIM-2}$ which is probably the most widely distributed MBL worldwide. *bla*$_{IMP-6}$ like *bla*$_{IMP-1}$ is found in both Japan and Korea but so far has not made it to Singapore (156, 186).

The nature of spread and implications for infection control

The spread of acquired carbapenemase genes may be the result of a number of different mechanisms. These include the spread of resistance determinants into the circulating bacterial population, or direct human-mediated dispersal of clones with properties that promote their spread. The distinction between the 2 is that clonal spread may be more rapid and driven by specific selection pressures like antibiotic use, whereas spread of resistance determinants continues at a steady pace even without selection as genes are apparently quite regularly exchanged between bacteria (187).

The outbreak strains of *P. aeruginosa* and *A. baumannii* seem to be related to the presence of specific clones. The *P. aeruginosa* clones from 2000 and the *A. baumannii* clones from 1996 did not persist suggesting that infection control measures based on limiting human-mediated spread may be useful, though in the case of *A. baumannii* it is also possible that they were outcompeted by global clones.

The situation in *Enterobacteriaceae* may be more complex. $bla_{\text{OXA-181}}$ seems to be mainly spread by a specific clone. On the other hand, $bla_{\text{KPC-2}}$, $bla_{\text{NDM-1}}$, and $bla_{\text{OXA-48}}$ are found on plasmids that freely conjugate. The plasmids bearing $bla_{\text{NDM-1}}$ are particularly promiscuous and when newly introduced to a population, the host bacteria are liable to show diversity. Inevitably however, some will eventually enter host bacteria that spread clonally as has happened

in the case with *bla*$_{KPC-2}$ (139). In a recent outbreak at a Canadian tertiary care centre, transmission of two clones of *bla*$_{NDM-1}$ –positive *K. pneumoniae* was terminated by implementation of contact precautions, screening of contacts, and environmental disinfection (188). In 2006, a nationwide clonal outbreak of *bla*$_{KPC-3}$-positive *K. pneumoniae* in Israel required a national response when local measures failed. The investigators in that outbreak attributed their success to a combination of standard precautions, contact isolation, and the assignment of dedicated nursing staff (189). There is less experience successfully controlling the spread of resistance plasmids as opposed to bacterial clones. It seems plausible that environmental cleaning and antimicrobial stewardship may have a more important role to play in these situations though there is precious little evidence to support any specific measure as being effective. However hospital infection control efforts will be to no avail if a large reservoir of resistance genes exists beyond the hospital boundary. In 2000, the same clone of IMP-1-producing *P. aeruginosa* was found in both acute and step-down community hospital in Singapore (59). Carbapenemase-producing *K. pneumoniae* have been also found in the community and in nursing home residents in other countries (190). We are not sure when *bla*$_{KPC-2}$ entered Singapore but the presence of a common PFGE type in patients with no epidemiological linkage in 3 different hospitals suggests there is a hidden reservoir that remains to be discovered (139).

Finally, it is worth taking a step back and evaluating the acquired carbapenemase that Singapore has arguably had the most experience with. *bla*$_{IMP-1}$-positive *K. pneumoniae* was first discovered in Singapore in 1996

(58). This was one of the first descriptions of an *Enterobacteriaceae* producing an acquired carbapenemase. *bla*$_{IMP-1}$ is also established in *P. aeruginosa* locally (59). When these results were first published, it was anticipated that a wave of CRE would follow (58). To a large extent, this has failed to materialize, despite the fact that *bla*$_{IMP-1}$ has clearly been circulating in the background all this time and the c.a. 150-160 kb plasmid carrying the gene seems quite capable of transferring between strains. There have been no local outbreaks of *bla*$_{IMP-1}$-positive *Enterobactericeae* and the number of isolates remains relatively small. The impact of this gene among *Enterobacteriaceae* at least is perhaps less than what was originally expected.

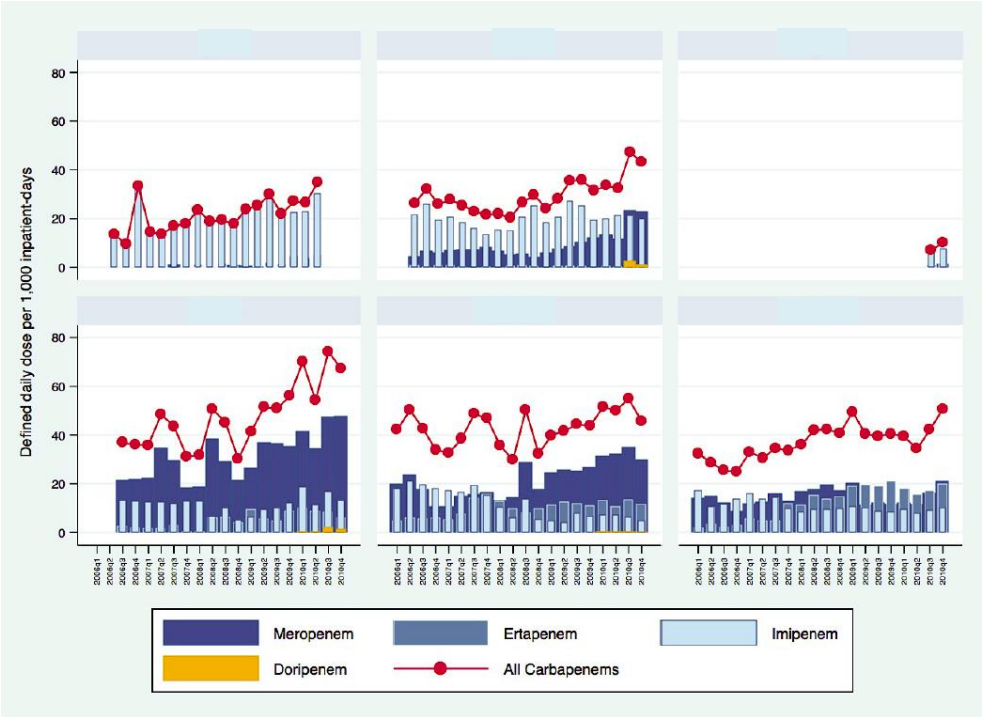
Unanswered questions and future work

The *bla*$_{NDM-1}$ and *bla*$_{OXA-181}$ genes are thought to have originated from *Acinetobacter* spp. (191) and *Shewanella xiamenensis* (192) respectively. Their entry into Singapore seems clearly linked to the importation of *Enterobactericeae* carrying these genes (149, 150). Paradoxically, less is known about the origins of *bla*$_{IMP-1}$. The environmental source of this gene still remains to be discovered. Was *bla*$_{IMP-1}$ imported into Singapore from Japan in the past or is there a common environmental source in these two countries? The presence of *bla*$_{IMP-1}$ in simple integrons in environmental Pseudomonads seems to point to the latter but the relatively limited distribution range of *bla*$_{IMP-1}$ to countries with geo-economic links to Japan argues for the former (60).

What makes some clones more successful at spreading globally than others? Why did some clones cause local outbreaks where others failed? Possible explanations that remain to be proven include stochasticity or genes/factors, other than antimicrobial resistance, that promote their survival and spread.

Finally, there is the question as to why this is all happening now. Imipenem was officially introduced to the Singapore General Hospital in June1998. This was followed by meropenem in February 2000, ertapenem in October 2003, and Doripenem in March 2012 (Personal communication Ms Winnie Lee, Department of Pharmacy, Singapore General Hospital). During this period, carbapenem usage has steadily increased exerting a corresponding antimicrobial pressure on the bacterial population. However this does not explain everything because increased carbapenem usage only led to a slight increase of carbapenem-resistant Acb and no increase of carbapenem-resistant *P. aeruginosa* (140).

Figure 30 Defined daily dose of carbapenems in 6 public hospitals, 2006-2010 (193).



Another contributing factor may be the increase in medical tourism. In 2007, Singapore received 571,000 foreign patients mainly from Indonesia, Malaysia and the Middle East (194) . Singapore had set a target of attracting 1 million foreign patients by 2012 (195). Given that many of these patients are likely to come from countries with high levels of antimicrobial resistance, Singapore must remain vigilant to avoid becoming a mixing vessel for antimicrobial resistance determinants and eventually a net exporter of multidrug-resistant bacteria. In addition, the majority of foreign patients attend private hospitals. The private sector therefore represents a potential ‘blind corner’ as the microbiology laboratories of these hospitals often run without doctorate-level microbiologists and are not so well-integrated with the public sector hospital

laboratories. There is a danger that in private laboratories, multidrug-resistant bacteria may either remain undetected or their significance unappreciated.

A complex picture emerges with interplay between antimicrobial usage, increased importation as a result of foreign travel and medical tourism, and the intrinsic properties of particular clones and transmissible resistance elements that favour spread. The future trend is likely to be bleak, especially for CRE. Some of the global clones of *Enterobacteriaceae* that carry carbapenemase genes are the same ones that harbour CTX-M ESBL genes. First described in the late 1980s, CTX-M-producing Gram-negative bacilli spread rapidly in the mid-1990s to become the dominant mechanism for oxyimino-cephalosporin resistance worldwide today (196). Both $bla_{\text{NDM-1}}$ and $bla_{\text{KPC-2}}$ have been found in the environment (37, 197). There is therefore the possibility that CRE, like CTX-M and pAmpC-producing *Enterobacteriaceae*, may eventually enter into the food chain where resistance is driven by even greater antimicrobial use (198, 199). This is important because some of the countries with significant problems with CRE (India and China) are also major exporters of raw food into Singapore (200). Future lines of research include determining which elements of spread are most important and the most effective infection control measures. The environmental reservoirs of some carbapenemase genes like $bla_{\text{IMP-1}}$ still remain to be discovered. The introduction of newer and even more detailed methods of typing like whole genome sequencing may reveal further insights into the spread of these organisms (201).

There are a number of weaknesses in the studies in this thesis. Firstly, the numbers of isolates were relatively small and in the cause of *P. aeruginosa* and *Enterobacteriaceae*, only one hospital was represented. However, Singapore is a small country with a mobile patient population, so the regional differences between one hospital and another may be less than in larger countries. Second, the isolates were not systematically collected but represented random collections at different time intervals. This was partly governed by the grants available at the time. Third, resources did not permit the collection of more clinical epidemiology data from the patients which would have helped correlate the molecular epidemiology work on the isolates. Finally, the *P. aeruginosa* and Acb work was done earlier and the situation may have changed. This is especially relevant for the newly introduced $bla_{\text{NDM-1}}$ and $bla_{\text{KPC-2}}$ which have been known to spread in these two species (202, 203). This illustrates the constantly changing landscape that is the field of antimicrobial resistance epidemiology and emphasizes the importance and continued need for surveillance. This should be done in a regular, systematic fashion fully funded at the national level. To achieve this, the Ministry of Health in Singapore has created a National Public Health Laboratory which tests for novel antimicrobial resistance determinants and a National Antimicrobial Taskforce which conducts surveillance of antimicrobial resistance of key pathogens. However the molecular typing of important multidrug-resistant bacteria is not routine. Given the known potential for antibiotic resistance determinants to enter into the food chain, veterinary and food surveillance would be prudent and there should be more co-operation

between the agencies responsible for animal, environmental, and human health.

Conclusion

The aim of this thesis was to identify the reasons behind the increase in carbapenem-resistance in Gram-negative bacilli in Singapore. The hypothesis was that this was the result of the introduction of global clones with the potential to cause outbreaks.

This hypothesis is only partially proven possibly because the situation may be in transition. For *P. aeruginosa* and *A. baumannii*, only local clones were present in the earlier isolates collected in 2000 and 1996 respectively. Global clones for *P. aeruginosa* (ST235) emerged in the isolates collected in 2008 but were heterogenous and did not cause local outbreak clusters. Furthermore, the carbapenemase-producers only represented less than 10% of *P. aeruginosa* that were resistant to imipenem suggesting that most of the carbapenem resistance in this species may be driven by other mechanisms like AmpC hyper-production in combination with porin loss. In summary, global clones of carbapenemase-producers have recently emerged in *P. aeruginosa* in Singapore but carbapenemase-producing *P. aeruginosa* did not pose a significant problem at the time of the study.

Global clones of *A. baumannii* (ST1 and ST2) were found in both the 2001 and 2006. Unlike *P. aeruginosa*, almost all carbapenem-resistant *A. baumannii* were carbapenemase-producers. In 2001, global clones of *A. baumannii* cause local outbreak clusters and at least one of these clones persisted in one hospital to 2006. Therefore global clones are likely to have played a role in the high rates of carbapenem-resistance among *A. baumannii* in Singapore.

Unlike the other 2 species, carbapenem-resistance in *Enterobacteriaceae* is a relatively new phenomenon. The situation is also confused by the ability of some of the carbapenemase genes to be easily transferred via plasmids. Nevertheless, some global clones were found (ST11 and 14 in *K. pneumoniae* and ST101 and ST405 in *E. coli*). Perhaps the reason why $bla_{\text{IMP-1}}$ has not become as widespread as originally anticipated is due to the fact that it is not commonly found in one of these global clones. While the numbers are presently relatively small, with $bla_{\text{KPC-2}}$, $bla_{\text{OXA-181}}$, and $bla_{\text{NDM-1}}$ found in global clones, an increase in CRE is likely in the future.

These studies show the diversity of acquired carbapenemase genes in Singapore, their distribution among different species, and the mechanisms underlying their spread. Little is known about the molecular epidemiology of multidrug resistance in Gram-negative bacilli in Southeast Asia. These studies have contributed to the general body of knowledge about the worldwide distribution of acquired carbapenemase genes by filling in this very important gap.

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Appendices

Appendix 1 Buffers for PFGE

1) PIV Buffer

1M Tris pH 8.0

1M Tris pH 8.0	60.55 g
Clinical Lab Reagent Water	400 ml
Concentrated HCl	~21 ml (adjust to pH)

PIV Buffer

1M NaCl	29.22 g
1M Tris, pH 8.0	1 ml
Clinical lab reagent water	500 ml

2) EC Buffer

0.5 M EDTA pH 8.0

$Na_2EDTA.2H_2O$	93.05 g

Clinical lab reagent water	400 ml
NaOH pellets	10 g

EC Lysis buffer

0.5 M EDTA pH 8.0	100 ml
1M Tris pH 8.0	3 ml
1M NaCl	29.2 g
Sodium deoxycholate	1 g
N-laurylsarcosine sodium salt	2.5 g
Clinical lab reagent water	~400 ml

<u>3) ES Buffer</u>

$Na_2EDTA.2H_2O$	93.1 g
Clinical lab reagent water	500 ml
NaOH pellets	~10 g
N-laurylsarcosine sodium salt	5 g

Appendix 2 Buffers for AFLP™

1) Lysis Buffer

Guanidine-iso-thiocyanate	120 g
0.1 M Tris-HCl (pH 6.4)	100 ml
0.2 M EDTA (pH 8.0)	22 ml
Triton X-100	2.6 g

Dissolve at 65ºC. Keep in the dark.

2) Wash Buffer

Guanidine-iso-thiocyanate	120 g
0.1 M Tris-HCl (pH 6.4)	100 ml

Dissolve at 65ºC. Keep in the dark.

Appendix 3 Comparison of Acb strains from Singapore with reference strains by AFLP.