Research Article

Detection of Antimicrobial Resistance Genes Associated with Carbapenem Resistance from the Whole-Genome Sequence of Acinetobacter baumannii Isolates from Malaysia

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Background. The spread of carbapenem-resistant A. baumannii (CrAb) is gaining worldwide attention. The spread of this pathogen is largely due to its ability to acquire various resistance genes of intrinsic and extrinsic origins that confer unpredictable susceptibility to β-lactams. The aim of this study was to analyze β-lactamase genetic compositions of CrAb in Malaysia. Methods. Whole-genome sequencing (WGS) was carried out on 13 CrAb isolates from clinical samples in Malaysia from 2011 to 2016. Results. Endotracheal aspirate was the dominant clinical sample source (n = 6), and only one isolate was obtained from wound swab. A total of 6 sequence types (STs) of the Oxford scheme were identified, including 4 reported STs and 2 novel STs. Eleven isolates were classified into clonal complex 92 (CC92/ICII), among which ST195 and ST208 were the most prevalent STs. All 13 CrAb isolates harbored multiple β-lactamase genes. blaOXA-23 (n = 13) and blaOXA-66 (n = 11) were the dominant carbapenemase gene families found in these isolates. All isolates harbor blaADC, blaOXA-51-like and blaOXA-23-like genes. blatem (n = 7), blaNDM-1 (n = 3), blaCARB-8 (n = 1), and blaper-3 (n = 1) are amongst other β-lactamase genes found in this study. ISAba1 was found upstream to blaOXA-23 (n = 13), blaOXA-66 (n = 1), and blaADC (n = 11). All blaNDM-1 isolates had ISAba125 (mobile genetic element) upstream to the genes. All isolates were positive for Tn2006/2008 and Tn2009 but were negative for Tn2007. Conclusion. Most of the isolates were grouped under the CC92 clonal complex which belongs to international clonal lineage 2. These findings predict that carriage of carbapenem-resistant genes possibly constitutes the underlying basis of high level of international clone II prevalence. Therefore, molecular surveillance and antimicrobial stewardship are essential in implementing policies to prevent and control the spread of CrAb in hospital settings.

1. Introduction

Acinetobacter baumannii (A. baumannii) is an infectious agent that has been the leading cause of hospital-acquired infections [1]. It is an opportunistic pathogen that poses significant threat to public health and associated with high mortality [2]. A. baumannii nosocomial infection is now common throughout the world [3, 4]. Selection of an appropriate empirical antimicrobial agent is extremely difficult due to its unpredictable antimicrobial resistance genes which are commonly acquired via mobile genetic elements [5].

A. baumannii belongs to a group of clinically important organism, known as ESKAPE. It is predominantly found among health care-associated organisms that have the potential of substantial antibiotic resistance [6]. A. baumannii infection usually involves excretory organ systems that contain high level of fluids. The most common sites of infection are respiratory tract, urinary tract, and peritoneal cavity and highly associated with indwelling devices such as endotracheal tube, urinary catheter, Tenckhoff catheter, and intravenous catheter [7].

Carbapenem-resistant A. baumannii (CrAb) was identified as the critical organism based on the global priority
pathogen list proposed by the World Health Organization (WHO). It has been concluded that development of new antimicrobial is the current focus globally [8]. CrAb has become a major concern among healthcare facilities due to its rising prevalence. In countries of the Arab League and Vietnam, prevalence of CrAb has been reported ranging from 50 to 88%, whereas in the United Kingdom, it ranges from 40 to 70% [9, 10]. According to the National Surveillance Antibiotic Resistance Database, CrAb prevalence in Malaysia ranges from 50 to 60% and remained static since year 2008 up to 2016 [11]. However, several studies from different hospitals in Malaysia showed CrAb prevalence higher than the national surveillance [12].

Nonjudicious use of antibiotics has led A. baumannii to rapidly acquire antimicrobial resistance genes from the environment. At the same time, selective antimicrobial pressure induces genome rearrangement associated with chromosomally (intrinsically) encoded antimicrobial resistance genes which has resulted in transposition of insertion sequence (IS) as a promoter of various CHDLs [13]. A. baumannii possesses bla\textsubscript{OXA-51-like}, an intrinsic carbapenem-hydrolysing oxacillinase gene. The expression of this gene may vary with the presence of IS\textsubscript{Abal} as a promoter [14]. It also acquires certain bla\textsubscript{OXA} and bla\textsubscript{non-OXA} group genes from plasmids [15]. Predominantly acquired bla\textsubscript{OXA-group} gene is bla\textsubscript{OXA-23-like} whereas the most prevalent bla\textsubscript{non-OXA} group gene is bla\textsubscript{NDM-1} [16].

This study is aimed to analyze the molecular characteristics of 13 A. baumannii isolates obtained from hospitalized patients in Malaysia with underlying carbapenem-resistant phenotype.

### 2. Methods

Of 1933 A. baumannii isolates collected from various hospitals throughout Malaysia from year 2011 to 2016, we selected 13 carbapenem-resistant A. baumannii (CrAb) isolates that were resistant to carbapenems. No genes (bla\textsubscript{NDM}, bla\textsubscript{OXA}, bla\textsubscript{KPC}, bla\textsubscript{VIM}, and bla\textsubscript{IMP}) were found using in-house PCR. These isolates were recovered from patients receiving intensive care and were isolated from respiratory secretion, urine, rectal swabs, and pus. The initial identification test based on biochemical methods was performed using API 20E (bioMérieux, LaPlane, France). Antimicrobial susceptibility was determined by the disc diffusion method for gentamicin, amikacin, ciprofloxacin, cefepime, ceftazidime, aztreonam, imipenem, meropenem, ertapenem, and ampicillin-sulbactam according to Clinical and Laboratory Standards Institute (CLSI) criteria. Minimum inhibitory concentrations (MICs) of imipenem, meropenem, and ertapenem were determined by the E-Test method according to CLSI criteria. CrAb is defined as an A. baumannii isolate that is resistant to meropenem, ertapenem, and imipenem with an MIC value of $\geq 4\, \mu g/ml$ via ETest. Table 1 summarizes study isolates, types of specimen, and the genebank identification.

Total DNA of these strains was extracted by using a MasterPure™ DNA Purification Kit (Epicentre, Madison, Wisconsin, USA) and quantified using Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA). DNA libraries were prepared using a Nextera DNA Flex Library Prep Kit (Illumina Inc.), according to the manufacturer’s instructions. Sequence data for all strains were obtained using an Illumina Nextseq platform (Illumina Inc., San Diego, CA, USA). Raw sequence quality trimming was carried out as described by SPAdes version 3.9.1 for de novo assembly and confirmation [17].

Average nucleotide identity (ANI) was calculated by using a gANI tool calculator, ANI calculator software version 1.0. ANI values above 95% between genomes of these isolates denote the same species [18]. Multilocus sequence typing (MLST) analysis was streamlined via the MLST program against PubMLST database via MLST version 2.6 software. Oxford scheme of A. baumannii was used for MLST analysis. The 7 housekeeping genes were glt\textsubscript{A}, gyr\textsubscript{B}, gdh\textsubscript{B}, rec\textsubscript{A}, cpn\textsubscript{60}, gpi, and rpo\textsubscript{D} [19]. New alleles and STs were submitted to the curator of the database, and new ST numbers were allotted. Clonal complexes were assigned by eBURST and were defined as single locus and double-locus variants with an outgroup A. baumannii strain ATCC 17978 as a reference (GenBank accession number: CP000521.1) [20]. Antimicrobial resistance genes (AMR) were confirmed by the CARD and resistance gene identifier (Resfinder) via Abricate-Version 0.8 software [21]. kSNP version 3.0 was

| Isolate        | Specimen type | NCBI biosample no. | GenBank accession no. |
|----------------|---------------|--------------------|----------------------|
| A. baumannii CRE1071/16 | Pus          | SAMN11513371       | SWLT00000000         |
| A. baumannii CRE1159/16 | Urine        | SAMN11513372       | SWLS00000000         |
| A. baumannii CRE157/16   | Urine        | SAMN11513373       | SWLR00000000         |
| A. baumannii CRE158/16   | Endotracheal aspirate | SAMN11513374 | SWLOQ00000000       |
| A. baumannii CRE245/15   | Rectal swab  | SAMN11513375       | SWLP00000000         |
| A. baumannii CRE341/15   | Urine        | SAMN11513376       | SWLOO00000000        |
| A. baumannii CRE400/16   | Endotracheal aspirate | SAMN11513377 | SWLNO00000000       |
| A. baumannii CRE449/14   | Endotracheal aspirate | SAMN11513378 | SWLMO00000000       |
| A. baumannii CRE596/14   | Endotracheal aspirate | SAMN11513379 | SWLI00000000         |
| A. baumannii CRE645/15   | Rectal swab  | SAMN11513380       | SWLK00000000         |
| A. baumannii CRE648/15   | Endotracheal aspirate | SAMN11513381 | SWJ00000000         |
| A. baumannii CRE85/16    | Urine        | SAMN11513382       | SWLI00000000         |
| A. baumannii CRE98/14    | Endotracheal aspirate | SAMN11513383 | SWLH00000000         |
used to identify pan-genome single-nucleotide polymorphism (SNP) [22]. A SNP phylogenetic tree was drawn based on pairwise whole-genome sequence via Type Genome Server using multiple reference strains that belong to the Acinetobacter baumannii complex group. Ugene-PRO and ISfinder applications were used to analyze the presence of mobile genetic elements [23]. The sequence of this whole-genome shotgun project has been deposited in GenBank under Genome submission: SUB5536145 with the BioProject ID: PRJNA539835.

3. Results

The genome sequence size of the 13 isolates in this study ranged from 3,832,120 to 4,246,682 base pair (bp), with contigs ranging from 70 to 104, which encodes 3577 to 4003 coding sequences, 50 to 53 tRNA, 3 rRNA, and 1 tmRNA. Six of 13 (46%) isolates were obtained from endotracheal aspirate followed by urine culture (4 (31%)), rectal swab (2 (15.4%)), and wound swab (1 (7.7%).) No isolate of CrAb was cultured from blood samples. Average nucleotide identity (ANI) of all isolates was above 95% which concludes that they belonged to the same species of bacteria. However, 3 paired isolates shared 100% identity of ANI although obtained from different sources and states. Those isolates were CRE400/16-CRE245/15, CRE645/16-CRE648/15, and CRE1071/16-CRE1159/16. Isolates from our study were compared with a genome of reference strain. The SNP-based phylogenetic tree showed that most of the isolate genomes were closely associated with each other and belonged to international clone II.

MLST analysis with the Oxford scheme in this study revealed a total of 4 defined STs and 2 novel STs (Figure 1). The 2 novel STs of year 2014-2015 were submitted and were assigned as ST1947 and ST1948. ST195, accounting for the largest proportion (5/13, 38%), was the major clonal type followed by ST208 (3/13, 23%), ST938 (2/13, 15.4), ST1418 (1/13, 7.7%), ST1947 (1/13, 7.7%), and ST1948 (1/13, 7.7%). Additionally, ST195, ST208, ST938, and ST1948 were double-locus variants of gyrB, gbbB, and gpi genes interchangeably. eBURST analysis showed that these 3 defined STs along with one novel ST clustered in the same CCs (CC92), which was also referred to as global clonal 2 (GC2)/ international clonal II (ICII).

All isolates harbored intrinsic blaOXA-51-like class D carbapenemases. blaOXA-66 was the most prevalent 11 (85%), followed by blaOXA-64 and blaOXA-91, 1 (8%) of each, respectively. An extrinsic blaOXA-type carbapenemase gene found was blaOXA-23 (100%), while no isolates contained blaOXA-24-like or blaOXA-58-like gene. Interestingly, all isolates
Table 2: Resistance genes present in carbapenem-resistant *A. baumannii* from year 2011–2016.

| Strain ID | State  | Source     | Oxford MLST | Clonal assigned | blaTEM | blaPER | blaOXA-23 | blaOXA-66 | blaOXA-91 | blaOXA-64 | blaNDM | blaADC | blaCARB | ISAbA1-ADC | ISAbA1-OXA23-like | ISAbA1-OXA51-like | Mobile genetic elements | Transposons |
|-----------|--------|------------|-------------|----------------|--------|--------|-----------|-----------|-----------|-----------|--------|--------|--------|------------|----------------------|----------------------|------------------------|-------------|
| CRE107/16 | Kelantan | Pus        | ST195       | CC92/IC2       | −      | −      | +         | +         | −         | −         | +      | +      | +      | +          | −                     | −                    | −                      | −            |
| CRE115/16 | Selangor | Urine      | ST195       | CC92/IC2       | −      | −      | +         | −         | −         | −         | +      | +      | +      | +          | −                     | −                    | +                      | −            |
| CRE157/16 | Perak   | Urine      | ST195       | CC92/IC2       | −      | −      | +         | −         | −         | −         | +      | +      | −      | −          | −                     | +                    | +                      | −            |
| CRE158/16 | Perak   | ETT        | ST195       | CC92/IC2       | +      | −      | +         | −         | −         | −         | +      | +      | +      | +          | −                     | −                    | +                      | −            |
| CRE245/15 | Kuala Lumpur | Rectal Swab | ST938      | CC92/IC2       | −      | −      | +         | −         | −         | −         | +      | +      | −      | −          | −                     | +                    | +                      | −            |
| CRE341/15 | Kuala Lumpur | Urine | ST1947      | CC229         | −      | +      | +         | −         | −         | −         | +      | +      | +      | −          | −                     | +                    | +                      | −            |
| CRE400/16 | Perak   | ETT        | ST938       | CC2/IC2        | +      | +      | +         | −         | −         | −         | +      | +      | +      | +          | −                     | −                    | +                      | −            |
| CRE449/14 | Pahang  | ETT        | ST1948      | CC92/IC2       | +      | −      | +         | −         | −         | +         | +      | −      | −      | +          | +                     | +                    | +                      | −            |
| CRE596/14 | Kuala Lumpur | Rectal Swab | ST208       | CC92/IC2       | +      | −      | +         | −         | −         | −         | +      | +      | −      | +          | −                     | +                    | +                      | −            |
| CRE645/15 | Kuala Lumpur | Rectal Swab | ST208       | CC92/IC2       | +      | −      | +         | −         | −         | −         | +      | +      | −      | +          | −                     | +                    | +                      | −            |
| CRE648/15 | Kuala Lumpur | Rectal Swab | ST208       | CC92/IC2       | +      | −      | +         | −         | −         | −         | +      | +      | −      | +          | −                     | +                    | +                      | −            |
| CRE683/16 | Pahang  | Urine      | ST418       | CC224         | −      | −      | +         | −         | +         | +         | −      | +      | +      | −          | −                     | +                    | +                      | −            |
| CRE99/14  | Sarawak | ETT        | ST195       | CC92/IC2       | +      | −      | +         | −         | +         | −         | +      | +      | +      | −          | −                     | +                    | +                      | −            |

*indicates the presence of the resistant gene. MLST sequence type (ST) along with clonal complex is included in the table. blaOXA, blaNDM, blaADC, blaTEM, and blaPER are the β-lactamase genes identified. IS: mobile genetic element-insertion sequence; Tn: transposon; ETT: endotracheal tube.
harbored more than one oxacillinase gene. Acinetobacter-
derived single-variant cephalosporinase blaADC gene was
present in all our isolates. A total of 8 (62.5%) isolates
harbored class A β-lactamase gene blaTEM, 1 isolate carried
blaCARB, and 1 isolate carried blaPER gene. In addition, 3
(23.1%) isolates carried class B metallo-β-lactamase (MBL)
gene blaNDM. Additionally, all isolates were negative for
other MBL genes which included blaIMP, blaVIM, blaGIM,
and blaSPM. Table 2 summarizes all the AMR genes detected
in this study.

As described in [24], we found that 1 (7.7%) isolate
harbors class 1 integron. The presence of mobile genetic
element provides strong evidence for the horizontal dis-
semination of antibiotic resistance genes. At the same time,
all blaOXA-23-like genes were carried by Tn2006 and Tn2008
in our study. In addition, we detect insertion sequence (IS)
elements in the promoter regions of several AMR genes [25].
All isolates with blaNDM gene were found to harbor
ISAba125 upstream to the corresponding gene blaNDM.
ISAba1 was found upstream to blaOXA-23 (n = 13), blaADC
(n = 11), blaOXA-66 (n = 1), and blaCARB (n = 1). IS91
was found upstream to blaPER (n = 1).

4. Discussion

A. baumannii of nosocomial origin has been the leading
cause of hospital-acquired infections [1]. The nature of this
bacterium is that it can be found in the environment, in-
trinsically carrying the antibiotic resistance gene and posing
a significant threat to public health due to its unpredictable
antibiotic susceptibility [2, 4, 5]. This study was aimed to
determine the genetic mechanisms conferring carbapenem
resistance in our local strains.

Most of the clinical isolates in this study obtained from
respiratory secretion (tracheal aspirate, sputum, and
bronchial alveolar lavage) were similar to the Malaysian
local surveillance study. Up to year 2017, nosocomial A.
baumannii was commonly isolated from respiratory se-
cretion, followed by blood isolation [11, 26]. Likewise,
many studies nationwide shared similar findings. A.
baumannii preferably colonizes or infects the respiratory
tract. Such infection commonly occurs in debilitated
patients especially in the ICU. Patients of mechanical
ventilation and lengthy hospital stay are at risk of A.
bau mannii infection [27, 28].
**Figure 3:** Geographic distribution of carbapenem-resistant *A. baumannii* strains according to different states in Malaysia from year 2011–2016.

ST195 was the frequent sequence types observed in our study. At the same time, as expected, most of these CrAb isolates belong to the CC92/IC2 clonal lineage. The predominance of CC92/IC2 in the present study was similar to reports produced in other neighbouring Asian countries such as China and Thailand and consistent with local studies and reports [29–31]. We also found novel strains of different clonal lineage emerging. Based on our study, these new strains have emerged in 2014 and 2015. In [19, 28, 32], the authors have revealed that emergence of newer strains is caused by inappropriate antibiotic usage. It is crucial to study about epidemiology of sequence types as there is horizontal dissemination and rapid spread [38].

In clinical microbiology laboratories, *A. baumannii* is indistinguishable with other species of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex by widely used routine identification systems due to its similar phenotypic and biochemical properties. The accurate identification of *A. baumannii* is only possible via molecular methods. Molecular characterization of *bla*<sub>OXA-51-like</sub> gene detection is carried out along with RNA polymerase β-subunit gene (*rpoB*) and DNA gyrase B gene (*gyrB*) for *A. baumannii* species identification [35]. All the isolates involved in this study were positive for *bla*<sub>OXA-51-like</sub>, *rpoB*, and *gyrB* genes. This finding resonates along with SNP phylogeny and ANI on species-level identification in this study.

*A. baumannii* is known for its enzymatic degradation mechanism by β-lactamases [36]. The most common carbapenem resistance mechanism found in our study was the existence of various β-lactamases and mobile genetic elements. *bla*<sub>OXA-23-like</sub> and *bla*<sub>OXA-51-like</sub> were the most prevalent, accounting for 100% carbapenem resistance amongst studied isolates. *bla*<sub>OXA-51-like</sub> gene was detected in all the isolates due to its chromosomal-borne nature, naturally occurring in oxacillinase gene [37].

Meanwhile, *bla*<sub>OXA-23-like</sub> gene can be either plasmid or chromosome-borne, resulting in increased rates of carbapenem resistance in healthcare settings due to its mobility in facilitating horizontal genetic transfer. The acquisition of *bla*<sub>OXA-23-like</sub> gene is a major public health concern for its horizontal dissemination and rapid spread [38].

No isolates harbored *bla*<sub>OXA-24-like</sub>, *bla*<sub>OXA-48-like</sub>, *bla*<sub>OXA-58-like</sub> genes. Although these genes are disseminated in Europe and Middle East, they remained rare in our local findings [39, 40]. A variant of *bla*<sub>OXA-51-like</sub> found in this study, namely, *bla*<sub>OXA-66</sub> is commonly found in China [41]. At the same time, the present study observed that both *bla*<sub>OXA-23-like</sub> and *bla*<sub>OXA-51-like</sub> genes are found in all the isolates. These findings were similar to [42] as it common to find *A. baumannii* isolates harbor *bla*<sub>OXA-23-like</sub> and *bla*<sub>OXA-51-like</sub> in the Asian continent, whereas *bla*<sub>OXA-51-like</sub> and *bla*<sub>OXA-58-like</sub> in the western hemisphere.

MBL carrying *A. baumannii* isolates are rare nationwide. *bla*<sub>NDM</sub> carrying *A. baumannii* is not commonly found in our area of study. We have not encountered *bla*<sub>NDM</sub> during previous years or among many local studies [38, 39]. However, to our surprise, a small number (3/13, 24%) of *A. baumannii* isolates collected during 2014–2016 were positive for *bla*<sub>NDM</sub>, indicating recent emergence. Worthy of mentioning is the fact that the isolates carrying *bla*<sub>NDM</sub> gene did not belong to the same clonal lineage [43]. This will be the first reporting on *bla*<sub>NDM</sub> gene harboring *A. baumannii* isolates from clinical samples in Malaysia.

Similar to the *bla*<sub>OXA-51-like</sub> oxacillinase gene, *bla*<sub>ADC</sub> is also a chromosomally encoded acinetobacter-derived
cephalosporinase gene found among all the A. baumannii isolates in this study. This finding indicates intrinsic-species specific gene [44]. blaTEM genes were also found in ¾ of our isolates. In [45], the authors had demonstrated that carbapenem resistance among CrAb is due to the coexistence of blaOXA-23 and blaTEM. Similar output was observed in this study; however, isolates without blaTEM also exhibit the resistant phenotypes. To a moderate level, blaCARB and blaPER were also detected. No prevalence of colistin-resistant genes was found among these isolates. However, there are A. baumannii isolates found in many case reports from neighbouring countries such as Taiwan, India, and China that are resistant and becoming resistant [46].

CRE341/15 isolate harbors integrase class 1 indicating sporadic clones. Isolates carrying mobile elements such as integron-encoded integrase gene flanking resistance genes are capable of acquiring and transferring virulence genes via recombination [47]. Transposon played a major role in dissemination of resistant genes. In this study, we observed the presence of transposon Tn2006/2008 in all the isolates carrying blaOXA-23 gene. This finding suggests that blaOXA-23 dissemination might be due to transposition of transposon [48]. In [14], the authors demonstrated that insertion of IS elements upstream to the resistant genes changes the expression level leading to the increased antimicrobial resistance phenotype. Every isolates in this study was found to have ISAba1 upstream to blaOXA-23, blaOXA-66, blaADC-7, and blaCARB. The ISAba125 was also found in the promoter region of all blanDM positive isolates. These findings suggest that the isolates may have other additional mechanisms resistance against carbapenem [49–51].

In summary, we demonstrated the possible clones of A. baumannii resistant to carbapenem and the prevalence of antibiotic resistance genes associated with mobile genetic elements. These findings provide epidemiological data of prevalent local STs as they are getting more diverse and resistant to multiple antibiotics. The presence of insertion sequence may reflect that these organisms readily take up external DNA. These findings are worrisome for its capability of outbreaks and horizontal resistance gene transmission. Molecular surveillance and antimicrobial stewardship are essential in implementing policies to prevent and control the spread of CrAb in hospital settings.

Data Availability

Genome database of this study is available in National Center of Biotechnology Information, NCBI.

Additional Points

**Highlights.** (i) The spread of carbapenem-resistant Acinetobacter baumannii is gaining global attention. (ii) It is an opportunistic pathogen that poses a significant threat to public health and is associated with high mortality. (iii) Selection of an appropriate empirical antimicrobial agent is extremely difficult due to its unpredictable susceptibility patterns. (iv) The association of a mobile genetic element with the resistant gene is worrisome and presents as an emerging threat to our healthcare settings.

**Ethical Approval**

This study obtained approval from the National Health Institute Human Research Ethics Committee.

**Conflicts of Interest**

All authors declare that they have no conflicts of interest.

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