



## The emergence of *Acinetobacter baumannii* ST-6 multidrug resistant: A resilient biofilm producer, Malaysia

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### ABSTRACT

**Aims:** The aim of this research is to explore the presence of multidrug-resistance (MDR) *Acinetobacter baumannii* strains isolated from hospitalized patients in a tertiary-care center, Subang Jaya, Selangor, Malaysia and to compare their genotypic and phenotypic characteristics.

**Methodology and results:** Clonal relationships were determined by multilocus sequence typing (MLST) and biofilm forming ability was evaluated by using 2, 3 - bis (2 - methoxy - 4 - nitro - 5-sulfohenyl) - 5 - [(phenylamino) carbonyl] - 2H-tetrazolium hydroxide (XTT) reduction assay in microplates and Congo red agar method (CRA). Four virulence genes coding for *A. baumannii* pilus usher-chaperone assembly protein, *csuE* gene; outer membrane protein, *ompA* gene; biofilm poly- $\beta$ -1, 6-Nacetylglucosamine (PNAG) synthesis protein, *pgaA* gene; and acinetobactin-mediated iron acquisition protein, *bauA* gene were searched for in a collection of strains. Antimicrobial resistance against 11 antibiotics were studied by broth microdilution method. Seventeen *A. baumannii* clinical strains were isolated and MLST showed that the strains belonged to 5 distinct sequence types (STs), namely, ST-6, ST-265, ST-324, ST-325 and ST-432. Fifty-three percent of the strains were resistant to 4 or more antibiotics. Twelve strains produced biofilm and out of them, 4 were strong biofilm producer, besides, these strong biofilm producers were MDR strains and belongs to ST-6. In addition, all strains were *ompA* positive, biofilm producing strains were *csuE* and *pgaA* positive and only strong biofilm producing strains were *bauA* positive.

**Conclusion, significance and impact study:** Our study demonstrates that the ST-6 strains in Malaysia could represent MDR, capable of forming strong biofilm and possess *csuE*, *ompA*, *pgaA* and *bauA* genes, virulence characteristics that probably help the bacteria to persist and cause infection.

**Keywords:** *Acinetobacter baumannii*, multilocus sequence typing, multidrug-resistant, biofilm

### INTRODUCTION

Multidrug-resistant (MDR) *A. baumannii* is a rapidly emerging pathogen in healthcare settings and their increasing incidence is an alarming problem. Management of MDR *A. baumannii* infections is an area of great concern for clinicians. This bacterium is responsible for a wide spectrum of infections that include pneumonia, bacteremia, meningitis, urinary tract infection and wound infection (Maragakis and Perl, 2008). The episodes of infections caused by MDR *A. baumannii* becomes more challenging when the strains have the ability to form biofilm. Biofilm is described as "a complex

aggregate of bacterial cells enclosed in a self-produced matrix of extracellular polymeric substance (EPS) adherent to an abiotic or biotic surface." It has been reported that *A. baumannii* can form biofilms on the surface which contribute to its ability to survive in a hospital setting and to persist for prolonged period (Manchanda *et al.*, 2010). The aim of this study was to explore the presence of MDR in a collection of *A. baumannii* isolated from patients and to compare the following genotypic and phenotypic characteristics: multilocus sequence typing (MLST), virulence-associated factor-encoding genes (*csuE*, *pgaA*, *ompA* and *bauA*), biofilm formation and resistance profiles to 11 frequently

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used antibiotics. This will assist our clinicians in prescribing an appropriate antibiotic against infections caused by MDR biofilm producer strains.

## MATERIALS AND METHODS

### Bacterial strains

Seventeen clinical *A. baumannii* strains were collected from hospitalized patients in a tertiary-care hospital in Subang Jaya, Selangor, Malaysia between 2012 and 2013. Identification of the species were confirmed by API 20-NE system (BioMérieux, France). The strains were stored in Luria-Bertani (LB) broth with 15% glycerol at  $-80^{\circ}\text{C}$  until further characterization.

### MLST

MLST based on the sequence of seven housekeeping genes (*gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi* and *rpoD*) were performed on all the strains as previously described (Qian *et al.*, 2015). Briefly, all seven genes were amplified and sequenced and allele number and ST were assigned according to the scheme proposed in the MLST website (<http://www.mlst.net/>).

### Resistotyping

All strains were tested for antibiotics resistance against a panel of 11 antimicrobials (polymyxin B, ampicillin/sulbactam, ceftazidime, meropenem, imipenem, amikacin, gentamicin, ciprofloxacin, levofloxacin, trimethoprim/sulfamethoxazole and tigecycline) using the broth microdilution following the guidelines recommended by Clinical and Laboratory Standards Institute guidelines (CLSI) M100-S24, 2014.

### Biofilm production assay

Overnight cultures of *A. baumannii* grown in LB broth

were washed twice with phosphate-buffered saline (PBS, pH 7.2) and diluted in PBS to obtain a suspension equivalent to 0.5 McFarland standards ( $\sim 1 \times 10^8$  CFU/mL). The suspension was then diluted to 1:100 in PBS. *Acinetobacter baumannii* ATCC 19606<sup>T</sup> was used as positive biofilm producing reference strains, whilst *Pseudomonas aeruginosa* ATCC 27853 was used as negative control. The experiment was performed in triplicate and repeated three times. Qualitative determination for the ability to form biofilm by the production of slime using Congo red agar (CRA) plate assay as described by Freeman *et al.*, 1989. The CRA medium is composed of 30 g/L Brain Heart Infusion (BHI) agar, 36 g/L sucrose and 0.8 g/L Congo red indicator. Sterilized aqueous Congo red stain was added to the media when the agar was cooled to  $55^{\circ}\text{C}$ . Plates were then inoculated with test strains and were incubated aerobically for 24 h at  $37^{\circ}\text{C}$ . Quantification of *A. baumannii* biofilms by XTT reduction assay was carried out by following the method described previously (Pierce *et al.*, 2010). Briefly, 100  $\mu\text{L}$  cell suspensions were inoculated into wells of sterile 96-well flat-bottomed polystyrene microtitre plates (©TPP, USA). The plates were incubated for 24 h at  $37^{\circ}\text{C}$  under static conditions. After incubation, each well was carefully washed three times with 200  $\mu\text{L}$  PBS, dried and 100  $\mu\text{L}$  of saturated XTT solution was added, the plates were covered with aluminium foil and incubated at  $37^{\circ}\text{C}$  for 2 h. After incubation, 80  $\mu\text{L}$  of the XTT supernatant was transferred to a fresh 96-well plate and the calorimetric changes were measured at 490 nm using iMark<sup>TM</sup> Microplate Absorbance Reader (Bio-Rad laboratories, Inc. USA). Results are reported according to published recommendations based on average optical density (OD) values and cut-off value (ODc) (ODc = average OD of negative control + [3  $\times$  standard deviation (SD) of negative control]) (Abdi-Ali *et al.*, 2014). Strains were divided into the following four categories: not a biofilm producer (OD  $\leq$  ODc); weak biofilm producer (ODc < OD  $\leq$  2  $\times$  ODc); moderate biofilm producer (2  $\times$  ODc < OD  $\leq$  4  $\times$  ODc); and strong biofilm producer (4  $\times$  ODc < OD).

**Table 1:** The sequences of the primers used with respective amplicon sizes in virulence characterization.

Genes	Primers	Amplicon size
<i>csuE</i>	Forward: 5' CTTGGCTTTGGCGAACAT 3' Reverse: 5' ACTGCGGGCACAGAATAG 3'	207
<i>pgaA</i>	Forward: 5' AGGTTGATGTATTGCGTGAG 3' Reverse: 5' TGCGGGTATTTATTTAGC 3'	100
<i>ompA</i>	Forward: 5' GACTACGGGCAAATCTCG 3' Reverse: 5' ACTGGCTCACGGCACCTT 3'	200
<i>bauA</i>	Forward: 5' TAACACGAGGCATCAACC 3' Reverse: 5' TTCCTTTTCAGCCCTACAT 3'	303

### Virulotyping

Virulence characterization on *A. baumannii* strains was performed by Polymerase Chain Reaction (PCR) in detection of *csuE*, *pgaA*, *ompA* and *bauA* genes associated in biofilm formation. Genomic DNA was

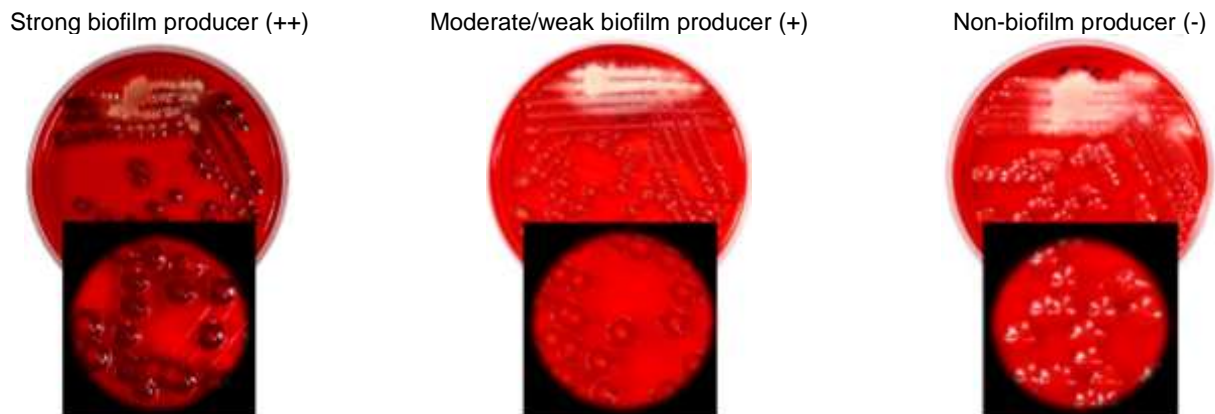
extracted using DNA Extraction Kit (GeneAll Biotechnology Co., Ltd, Korea) following the manufacturer's guidelines. The DNA sequence of *csuE*, *pgaA*, *ompA* and *bauA* s were obtained from the National Centre for Biotechnology Information (NCBI) GenBank (<http://www.ncbi.nlm.nih.gov>). Primer sequences were

designed using Primer Premier 3 and were synthesized by Sigma Aldrich (Table 1). Annealing temperatures were optimized by gradient PCR and cycling conditions for each primer were as follows: initial denaturation (95 °C) for 3 min; 35 cycles-denaturation (95 °C) for 30 sec, annealing (60 °C) for 30 sec and extension (72 °C) for 1 min; final extension (72 °C) for 10 min. All the amplified products were separated by electrophoresis in 1.3% agarose gels stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Inc., USA). The agarose gel was viewed under UV light using imaging system (AlphaImager® HP, USA).

## RESULTS

A total of 17 *A. baumannii* strains were tested in our study (Table 2). The strains were isolated from various body sites of infections from patients. Majority of the isolates were from wound swab ( $n = 10$ ), followed by respiratory sites include sputum and tracheotomy site ( $n = 6$ ) and blood ( $n = 1$ ). MLST showed that the 17 strains belonged to five distinct STs: ST-6, ST-265, ST-324, ST-325 and ST-432. Fifty-three percent of the *A. baumannii* strains were resistant to at least four of the eleven tested antibiotics: imipenem, meropenem, gentamicin and amikacin. Likewise, four strains were resistant to nine out of the eleven antibiotics tested: ampicillin/sulbactam,

ceftazidime, imipenem, meropenem, gentamicin, amikacin, ciprofloxacin, levofloxacin and trimethoprim/sulfamethoxazole. These four strains were classified as MDR [strains resistant to at least three classes of antimicrobial agents - all penicillins and cephalosporins (including inhibitor combinations), fluoroquinolones, and aminoglycosides] (Manchanda *et al.*, 2010). All the MDR strains were belonged to ST-6. A good correlation was observed between the MLST results and antibiotic resistant pattern. Of them, 4 strains showed black colonies (strong biofilm producer), 8 strains showed black colonies with red center (moderate/weak biofilm producer) and 5 strains showed red colonies (non-biofilm producers) (Figure 1). These CRA results were validated with XTT reduction assay. XTT reduction assay showed that 12 strains were biofilm producer (4 strains - strong biofilm producer; 4 strains - moderate biofilm producer; 4 strains - weak biofilm producer). All *A. baumannii* strains tested positive for the *ompA* gene, which codes for outer membrane protein of *A. baumannii*. Conversely, all biofilm producing strains harbored the *csuE* and *pgaA* genes, coding for pilus usher-chaperone assembly protein and biofilm PNAG synthesis protein respectively. Interestingly, only strong biofilm producer strains tested positive for *bauA* gene, coding for acinetobactin-mediated iron acquisition protein. In addition, these strong biofilm producer were of the MDR strains.



**Figure 1:** Colony morphologies of *A. baumannii* clinical strains on CRA.

## DISCUSSION

*Acinetobacter baumannii* has emerged as leading opportunistic pathogens and nosocomial pathogen worldwide (Allen and Hartman, 2009). MDR *A. baumannii* emergence could therefore complicate the treatment, leading to recurrent disease. Four of the seventeen tested strains (23.5%) were MDR. According to MLST results, these four MDR strains were belong to ST-6. To our knowledge, this is the first report of the ST-6 in Malaysia, ST-6 was originally reported to belong to Barcelona epidemic strains that was responsible for the outbreak in Elche General Hospital during 2001 and 2002 (Bartual *et al.*, 2005). In addition, Bartual *et al.* (2005) has also reported that the ST-6 to be a clonal lineage of the

European clone II, which highlights the widespread of this successful epidemic clone during the past two decades. The MDR *A. baumannii* appears to play a significant role in the remarkable ability to persist and spread in the hospital settings, together with its ability to colonize both abiotic and biotic surfaces and to grow as biofilm has been studied (Eijkelkamp *et al.*, 2011; Gurung *et al.*, 2013). In agreement with previously published results on MDR *A. baumannii*, we found that MDR *A. baumannii* are good biofilm producers (Eijkelkamp *et al.*, 2011; Gurung *et al.*, 2013). This feature could confer advantages in persistence and spreading of MDR *A. baumannii* ST-6 in the hospital settings. Concerning virulotyping, all strains possessed the *ompA* gene, however only biofilm producing strains possessed *csuE*, *pgaA* and surprisingly

**Table 2:** *Acinetobacter baumannii* strains used in the present study and their characteristics.

Strain	Year of isolation	Source	ST	Resistotype											Biofilm Phenotype		Virulotype <sup>d</sup>			
				AMS	CAZ	IMI	MRP	PB	CN	AK	TGC	CIP	LEV	SXT	CRA <sup>b</sup>	XTT reduction assay <sup>c</sup>	<i>csuE</i>	<i>pgaA</i>	<i>ompA</i>	<i>bauA</i>
AB-1	2012	Sputum	265	S	S	R	R	S	R	R	S	S	S	S	-	-	-	-	+	-
AB-2	2012	Sputum	265	S	S	R	R	S	R	R	S	S	S	S	-	-	-	-	+	-
AB-3	2012	Sputum	265	S	S	R	R	S	R	R	S	S	S	S	-	-	-	-	+	-
AB-4	2012	Wound	325	S	S	S	S	S	S	S	S	S	S	S	+	++	+	+	+	-
AB-5	2012	Sputum	265	S	S	R	R	S	R	R	S	S	S	S	-	-	-	-	+	-
AB-6	2012	Wound swab	325	S	S	S	S	S	S	S	S	S	S	S	+	++	+	+	+	-
AB-7	2012	Wound swab	6	R	R	R	R	S	R	R	S	R	R	R	++	+++	+	+	+	+
AB-8	2012	Wound swab	6	R	R	R	R	S	R	R	S	R	R	R	++	+++	+	+	+	+
AB-9	2012	Wound swab	325	S	S	S	S	S	S	S	S	S	S	S	+	++	+	+	+	-
AB-10	2012	Wound swab	432	S	S	S	S	S	S	S	S	S	S	S	+	+	+	+	+	-
AB-11	2012	Blood	324	S	S	S	S	S	S	S	S	S	S	S	+	+	+	+	+	-
AB-12	2012	Wound swab	432	S	S	S	S	S	S	S	S	S	S	S	+	+	+	+	+	-
AB-13	2012	Tracheotomy site	6	R	R	R	R	S	R	R	S	R	R	R	++	+++	+	+	+	+
AB-14	2013	Wound swab	6	R	R	R	R	S	R	R	S	R	R	R	++	+++	+	+	+	+
AB-15	2013	Wound swab	432	S	S	S	S	S	S	S	S	S	S	S	+	+	+	+	+	-
AB-16	2013	Sputum	265	S	S	R	R	S	R	R	S	S	S	S	-	-	-	-	+	-
AB-17	2013	Wound swab	325	S	S	S	S	S	S	S	S	S	S	S	+	++	+	+	+	-

<sup>a</sup>Resistotype against a panel of 11 known antibiotics were determined using the broth microdilution method. AMS, ampicillin/sulbactam; CAZ, ceftazidime; IMI, imipenem; MRP, meropenem; PB, polymyxin B; CN, gentamicin; AK, amikacin; TGC, tigecycline; CIP, ciprofloxacin; LEV, levofloxacin; SXT, trimethoprim/sulfamethoxazole; S, susceptible; R, resistant.

<sup>b</sup>Slime producing ability by CRA were determined. -, non-biofilm producer; +, weak/moderate biofilm producer; ++, strong biofilm producer.

<sup>c</sup>Biofilm forming ability by XTT reduction assay were determined. -, non-biofilm producer; +, weak biofilm producer; ++, moderate biofilm producer; +++, strong biofilm producer.

<sup>d</sup>PCR results using primers *csuE*, *pgaA*, *ompA* and *bauA* genes. -, absent; +, presence.

only strong biofilm producers possessed *bauA* genes. This *bauA* gene coding for acinetobactin-mediated iron acquisition protein may also help the bacteria to be more virulent and to persist. CRA method to detect biofilm production was performed in this study. It is known that Congo red can directly interact with certain polysaccharide forming colored complexes. In the present study, 12 (70.6%) of the total *A. baumannii* isolates showed biofilm production by CRA method which was verified and supported by the most reproducible, accurate and efficient XTT reduction assay (Taff *et al.*, 2012). The use of rapid, simple, inexpensive method like CRA method is thus an urgent priority for determining the biofilm producing ability which is feasible for low resources countries. Microbial biofilms lead to chronic infections and are a major challenge for the clinicians and have economic significance. Hence, detection of biofilm producers, judicious antibiotic usage and strict appliance of hospital infection control practices can assist in the prevention of such hitches.

In conclusion, this study provides an insight into MDR *A. baumannii* by demonstrating that: (i) ST-6 could represent MDR *A. baumannii* in Malaysia; (ii) All MDR *A. baumannii* in our study are strong biofilm producers and possess the *bauA* gene coding for acinetobactin-mediated iron acquisition protein.

#### CONFLICT OF INTEREST

None to declare.

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