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Original Article

Biocide susceptibilities and biofilm-forming capacities of *Acinetobacter baumannii* clinical isolates from Malaysia

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Abstract

Introduction. *Acinetobacter baumannii* is a Gram-negative nosocomial pathogen that has the capacity to develop resistance to all classes of antimicrobial compounds. However, very little is known regarding its susceptibility to biocides (antiseptics and disinfectants) and capacity to form biofilms, particularly for Malaysian isolates.

Aim. To determine the susceptibility of *A. baumannii* isolates to commonly-used biocides, investigate their biofilm-forming capacities and the prevalence of biocide resistance and biofilm-associated genes.

Methodology. The minimum inhibitory concentration (MIC) values of 100 *A. baumannii* hospital isolates from Terengganu, Malaysia, towards the biocides benzalkonium chloride (BZK), benzethonium chloride (BZT) and chlorhexidine digluconate (CLX), were determined by broth microdilution. The isolates were also examined for their ability to form biofilms in 96-well microplates. The prevalence of biocide resistance genes *qacA*, *qacE* and *qacΔE1* and the biofilm-associated genes *bap* and *abaI* were determined by polymerase chain reaction (PCR).

Results. Majority of the *A. baumannii* isolates (43%) showed higher MIC values (> 50 µg/mL) for CLX than for BZK (5% for MIC > 50 µg/mL) and BZT (9% for MIC > 50 µg/mL). The *qacΔE1* gene was predominant (63%) followed by *qacE* (28%) whereas no isolate was found harbouring *qacA*. All isolates were positive for the *bap* and *abaI* genes although the biofilm-forming capacity varied among the isolates.

Conclusion. The Terengganu *A. baumannii* isolates showed higher prevalence of *qacΔE1* compared to *qacE* although no correlation was found with the biocides' MIC values. No correlation was also observed between the isolates' biofilm-forming capacity and the MIC values for the biocides.

Key words: *Acinetobacter baumannii*; biocides; biofilm; chlorhexidine digluconate; benzalkolium chloride; benzethonium chloride.

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Introduction

Acinetobacter baumannii, a Gram-negative, non-fermentative coccobacilli, is a predominant cause of nosocomial infection and can survive on inanimate surfaces for long periods of time. It has gained prominence in the past few decades due to its ability to acquire and develop resistance to all classes of antibiotics over a relatively short period of time [1,2]. *A. baumannii* is estimated to be responsible for about 10% of nosocomial infections and has been identified as a cause of increased mortality of up to 80% in neonates, particularly in intensive care units [3,4]. In Malaysia, *A. baumannii* is the most prevalent nosocomial pathogen in most intensive care units [5–8].

Both the hospital environment and colonized patients have been shown to be major reservoirs for *A. baumannii* infections [9], thereby making the management and effective control of *A. baumannii* a challenge for hospital physicians and authorities. Biocides, including disinfectants and antiseptics, play a crucial role in the prevention of nosocomial transmission of infectious pathogens with the biguanide compound, chlorhexidine gluconate (CLX), and the quaternary ammonium compounds, benzethonium chloride (BZT) and benzalkonium chloride (BZK), being among the more extensively used [10]. However, extensive use of these biocides in hospitals had led to concerns on the development of resistance and the

spread of biocide resistance genes [10,11], mirroring the spread of antibiotic resistance in pathogenic bacteria. Despite the importance of biocide resistance, there is a dichotomy in the number of published papers regarding reduced susceptibilities to biocides when compared with the much larger volume of publications on antibiotic resistance [12]. In Malaysia, there has only been a single published paper so far that reported on antiseptic resistance in *A. baumannii* and its associated resistance genes [13].

Resistance to antiseptics and disinfectants in pathogenic bacteria are largely mediated by efflux proteins encoded by *qac* genes. The *qacA/B* genes encode for proteins of the major facilitator superfamily (MFS) whereas *qacC*, *qacE*, *qacF*, *qacG*, *qacH*, *qacJ* and *qacZ* encode for efflux proteins of the small multidrug resistance (SMR) family [14]. The *qacE* gene and its functionally active deletion derivative designated *qacΔE1*, are mainly found in Gram-negative bacteria, including *A. baumannii* [11]. These genes are commonly located on mobile elements such as integrons and transposons as well as transmissible plasmids, thus facilitating their spread [15].

The ability to form biofilms is one of the important virulence factors that enable *A. baumannii* to survive in the harsh hospital environment by affording the bacteria greater protection against antimicrobials and survival in dry and desiccated conditions [16]. *A. baumannii* is known to form biofilm communities on most abiotic surfaces and thus contributes to medical-device-associated infections [17]. Biofilm formation is a complex process involving a repertoire of genes and although several factors that contribute to biofilm formation appear to be strain-dependent, some common factors have been identified [16–18]. *A. baumannii* produce biofilm-associated proteins (Bap), which are large surface-exposed proteins secreted through a type I secretion system (T1SS), and plays an important role in cell-cell adhesion and the development of higher order structures on medically-relevant materials [17,19]. Biofilm formation in *A. baumannii* is also under the control of an auto-inducing quorum sensing molecule (acyl-homoserine lactone) that is biosynthesized by the *abaI*-encoded autoinducer synthase [16,20].

We have previously characterised *A. baumannii* isolates from the main tertiary hospital in the east coast state of Terengganu in Peninsular Malaysia for their antimicrobial resistance profiles and carriage of carbapenem resistance genes [21,22]. Here, we examine a sample of these isolates obtained from 2011 – 2016 for their susceptibilities to the biocides CLX, BZT and BZK and determine the prevalence of the biocide resistance genes *qacA*, *qacE* and *qacΔE*. We also investigate the biofilm-forming capacities of these *A. baumannii* isolates and their carriage of the biofilm-associated genes *bap* and *abaI*. This would give us a more well-informed picture of the Terengganu *A. baumannii* isolates, filling in the gap in our knowledge as data regarding biocide susceptibility and biofilm capability of *A. baumannii* from Malaysia are scarce.

Methodology

Bacterial strains

A total of 100 *A. baumannii* isolates used in this study were collected from the Microbiology Laboratory, Department of Pathology, Hospital Sultanah Nur Zahirah (HSNZ), Kuala Terengganu, Terengganu in 2011 (n = 6), 2012 (n = 14), 2015 (n = 30) and 2016 (n = 50). The isolates were identified as

Table 1. Detailed characteristics of some of the *A. baumannii* isolates from Hospital Sultanah Nur Zahirah (HSNZ), Terengganu, Malaysia (please refer to Supplementary Table 1 for the characteristics of all 100 *A. baumannii* isolates).

Isolate no.	Year of isolation	Ward	Source of isolate	Carbapenem resistance*		MDR status**	Antiseptic MIC (µg/mL)			*qacA*	*qacE*	*qacΔE1*	Biofilm formation		*bap*	*abaI*
				IMP[a]	MEM[b]		BZK[c]	BZT[d]	CLX[e]				OD_{540}	Interpretation***		
AB1202	2012	ICU	Urine	R	R	MDR	12.5	12.5	>50.0	–	+	+	1.160	Strong	+	+
AC1605	2016	ICU	Pus	R	R	MDR	25.0	>50.0	>50.0	–	–	+	0.242	Weak	+	+
AC1618	2016	Medical	ETT[f]	R	R	MDR	>50.0	>50.0	25.0	–	–	–	0.175	Weak	+	+
AC1622	2016	Medical	Blood	S	S	Non-MDR	12.5	12.5	>50.0	–	–	–	1.097	Strong	+	+
AC1624	2016	Medical	Pus	R	R	MDR	25.0	>50.0	>50.0	–	–	–	0.387	Moderate	+	+
AC1626	2016	Medical	Blood	R	R	MDR	12.5	6.25	25.0	–	+	+	0.504	Moderate	+	+
AC1629	2016	Medical	Pus	R	R	MDR	12.5	12.5	25.0	–	+	+	0.219	Weak	+	+
AC1636	2016	ICU	Pus	R	R	MDR	12.5	25.0	>50.0	–	–	+	1.069	Strong	+	+
AC1642	2016	ICU	ETT[f]	R	R	MDR	25.0	25.0	25.0	–	–	+	1.049	Strong	+	+

[a]IMP – imipenem; [b]MEM – meropenem; [c]BZK – benzalkolium chloride; [d]BZT – benzethonium chloride; [e]CLX – chlorhexidine gluconate; [f]ETT – endotracheal fluid/aspirate; *Carbapenem resistance (R = resistant; S = susceptible) as determined by disc diffusion using Clinical and Laboratory Standards Institute (CLSI) guidelines [23]; **Multidrug-resistance (MDR) status as defined by Magiorakos *et al*. [24], i.e., resistance to three or more classes of antimicrobials; ***Interpretation of OD_{540} values: < 0.3 = weak; between 0.3 – 1.0 = moderate; > 1.0 = strong.

Acinetobacter spp. by the hospital laboratory and validated as *A. baumannii* by *rpoB* sequencing as previously described [22]. Details of the source for the isolates are in Supplementary Table 1. There were fewer isolates from 2011 and 2012 due mainly to the intermittent collection of isolates in those years and a few them were unable to be revived from frozen stock cultures. Isolates from 2015 and 2016 were randomly chosen from our collection of *A. baumannii* isolates obtained from HSNZ during those two years. HSNZ is the main public tertiary referral hospital in the state of Terengganu, Malaysia and has a total of 821 beds and 29 wards with a 20-bed intensive care unit (ICU). Ethical approval for this study was obtained from the Medical Research & Ethics Committee of the Malaysian Ministry of Health's National Medical Research Register (approval no. NMRR-14-1650-23625-IIR).

Determination of MIC values for antiseptics

The antiseptics used in this study were the quaternary ammonium compounds, benzalkolium chloride (BZK) and benzethonium chloride (BZT) as well as the biguanide compound, chlorhexidine digluconate (CLX), which were purchased from Sigma-Aldrich (St. Louis, USA). A stock solution of the relevant antiseptic (100 mg/l) was prepared in sterile deionized water and stored at 4ºC. The minimum inhibitory concentration (MIC) of each antiseptic was determined using the broth microdilution method in sterile, disposable 96-well microplates as per the Clinical and Laboratory Standards Institute guidelines [23]. In the absence of any standard breakpoints for antiseptics against *Acinetobacter* spp., we decided to use concentrations that were modified from those reported by Babaei *et al*. [13]. Into wells one to twelve of a 96-well microplate, a 50 µL bacterial suspension in LB broth at 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL) was added. Subsequently, 50 µL of the stock antiseptic solution was added into well one, mixed with the bacterial suspension, after which 50 µL was then transferred to the next well and continued until the last well of that row. This would lead to the microplate row containing antiseptic concentrations that ranged from 50 to 0.024 µg/mL. For each test plate, two antiseptic-free controls were prepared: one containing 100 µL medium alone (acting as a sterility control) and another with 50 µL medium plus 50 µL of bacterial inoculum (as a growth control). The microplate was covered and incubated at 37ºC for 16 – 20 hours after which the turbidity was measured at 625 nm in a microplate reader. The MIC was defined as the lowest concentration of the antiseptic that inhibits visible growth of the tested *A. baumannii* isolate as measured by the OD_{625} values compared with the controls [12].

Antimicrobial susceptibility profiles

Susceptibility of the *A. baumannii* isolates to carbapenems [imipenem (10 µg) and meropenem (10 µg)] and 11 other antimicrobials [i.e., amikacin (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), piperacillin-tazobactam (100/10 µg), ticarcillin-clavulanate (75/10 µg), ampicillin-sulbactam (10/10 µg), cefotaxin (30 µg), ceftriaxone (30 µg), ceftazidime (30 µg), cefepime (30 µg), and tetracycline (30 µg)] was determined by the disc diffusion method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines [23]. Isolates that were resistant to three or more classes of antimicrobials were categorised as multidrug resistant (MDR) following the proposed criteria of the joint initiative of the European Centre for Disease Prevention and Control (ECDC) and the United States Centers for Disease Prevention and Control (CDC) [24].

Biofilm Assay

The capacity of the *A. baumannii* isolate to form biofilms was assayed following the method described by King *et al*. [25]. Each *A. baumannii* isolate was inoculated in 10 mL LB broth and incubated overnight at 37ºC. A 50 µL aliquot of the overnight culture was added into 50 µL LB broth in the well of a 96-well microplate and further incubated at 37ºC for 16 – 20 hours. The assay was performed in triplicates for each *A. baumannii* isolate. Following overnight incubation, the wells of the plate were washed four times with deionized H_2O, 100 µL of 0.1% crystal violet was added to each well and incubated at room temperature for 30 min. The plate was then washed four times with deionized H_2O before adding 200 µL 95% ethyl alcohol after which 125 µL of the sample was transferred to a new plate and the absorbance measured at 540 nm using a microplate reader. A reading of < 0.3 was considered as weak biofilm formation, between 0.3 – 1.0 was moderate, while readings of > 1.0 was considered as strong.

Genomic DNA extraction and polymerase chain reaction (PCR) of target genes

Genomic DNA from the *A. baumannii* isolates were extracted using the MasterPure DNA Purification Kit (Epicentre, Madison, WI, USA) according to the manufacturer's instructions. The genomic DNA

obtained was stored at -20ºC until used. PCR was used to screen all *A. baumannii* isolates for the presence of the quaternary ammonium compound resistance genes *qacA*, *qacE* and *qacΔE1*, and the biofilm-associated genes *bap* and *abaI*. Each PCR reaction consist of 25 µL of *Taq* DNA polymerase master mix RED 2.0x (Ampliqon, Odense, Denmark) to which was added 21 µL sterile distilled water and 1 µL each of the forward and reverse primers (at 20 nM each) and 2 µL genomic DNA (~50 ng/µL) to make up a total volume of 50 µL. The primer sequences and PCR conditions are listed in the Supplementary Table 2. The PCR-amplified products were detected by electrophoresis on a 1.5% agarose gel which was then stained with ethidium bromide and visualised under UV illumination in a transilluminator (Uvitek, Cambridge, UK). The amplicons obtained were purified using the GeneJet PCR Purification Kit (ThermoScientific, Waltham, USA) and sequenced using conventional Sanger dideoxy sequencing at a commercial DNA sequencing service provider (Apical Scientific, Seri Kembangan, Malaysia) for validation. DNA sequence data was analysed by BLAST at https://blast.ncbi.nlm.nih.gov/Blast.cgi.

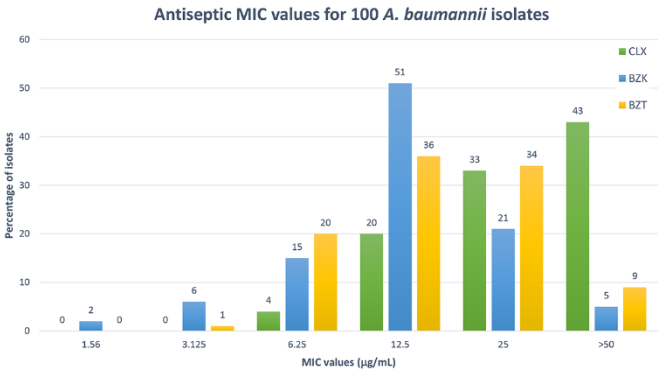
Statistical analyses

SPSS version 23.0 was used for the statistical analyses of the data obtained. Descriptive statistics were used to describe the differences in variables. Frequency and percentages were applied to characterise the prevalence of antiseptic resistance and biofilm-associated genes from *A. baumannii* that were isolated from different wards of the hospital. Chi-square was applied to determine correlations between the carriage of antiseptic resistance genes and observable reduced antiseptic susceptibility as well as between the carriage of biofilm-associated genes and extent of biofilm formation. *P* values < 0.05 were considered as statistically significant.

Results

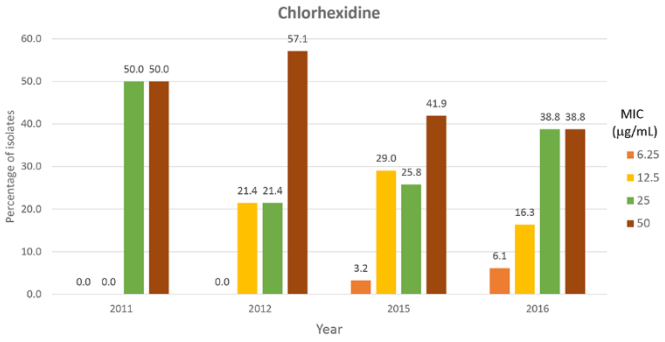
A significant proportion of the 100 *A. baumannii* isolates from HSNZ, Terengganu, showed higher MIC values (> 50 µg/mL) for CLX (43%) as compared to BZT (9%) and BZK (5%) (see Supplementary Table 1 for the full results of the 100 isolates). Slightly over 50% of the *A. baumannii* isolates had an MIC value of 12.5 µg/mL for BZK whereas for BZT, the isolates mainly displayed MIC values of 12.5 µg/mL (36%) and 25 µg/mL (34%) (Figure 1). When the results were analysed according to the year of isolation, the number of *A. baumannii* isolates with a high CLX MIC of > 50 µg/mL showed a decline from a high of 57.1% in 2012 to 38.8% in 2016 (Figure 2). Isolates with a lower CLX MIC value of 6.25 µg/mL also appeared in 2015 and 2016. In contrast, the prevalence of isolates with high MIC values for BZT (i.e., 25 µg/mL and > 50 µg/mL) showed a steady increase from 2011 to 2016 with isolates displaying an MIC value of 50 µg/mL only appearing in 2016 (Figure 3A). Likewise, an increase in the prevalence of isolates with higher MIC values for BZK was also observed with those showing an MIC value of > 50 µg/mL also appearing only in 2016 (Figure 3B). However, it should be noted that the number of *A. baumannii* isolates from 2011 (n = 6) and 2012 (n = 14) were smaller as compared to 2015 (n = 30) and 2016 (n = 50), and thus any comparisons should be made with caution.

Figure 1. Biocide MIC values for the Terengganu *A. baumannii* isolates.



MIC values for chlorhexidine gluconate (CLX), benzethonium chloride (BZT) and benzalkolium chloride (BZK) of the 100 *A. baumannii* strains from Hospital Sultanah Nur Zahirah (HSNZ), Terengganu, Malaysia, obtained from 2011 – 2016. The percentage of isolates having the respective MIC values is indicated above the respective bars of the chart.

Figure 2. The percentage of the Hospital Sultanah Nur Zahirah (HSNZ) *A. baumannii* isolates with the respective MIC values for chlorhexidine gluconate according to the year of isolation.



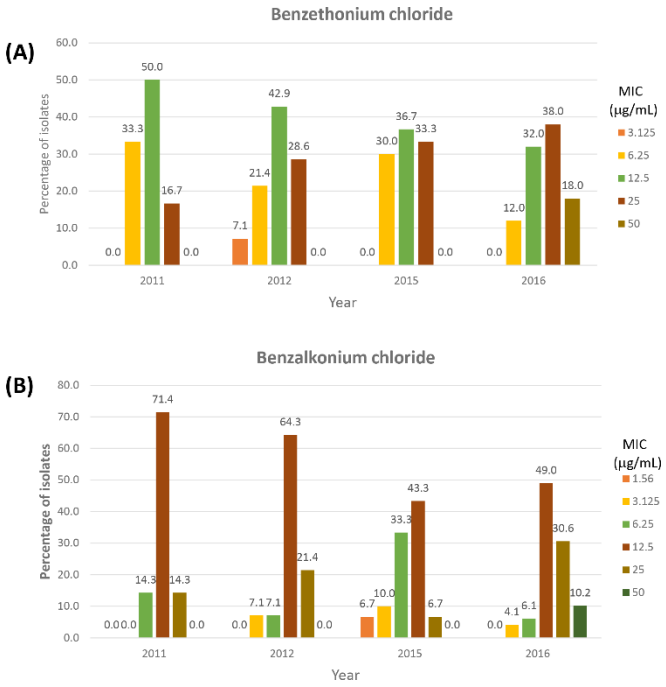
The *qacΔE1* gene was predominant (63%) followed by *qacE* (28%) whereas none of the HSNZ *A. baumannii* isolates harboured the *qacA* gene. No significance ($p > 0.05$) was observed between carriage of either the *qacΔE1* or *qacE* gene with high MIC values for CLX, BZK and BZT. Isolates such as AC1626 and AC1629, which were positive for both *qacΔE1* and *qacE*, yielded CLX MIC of 25 µg/mL and BZK and BZT MIC values of 12.5 µg/mL (Table 1). Conversely, isolates such as AC1618 and AC1624, which were negative for both *qacΔE1* and *qacE*, showed higher MIC values for CLX (> 50 µg/mL) as well as BZT and BZK (either 25 µg/mL or > 50 µg/mL) (Table 1).

All 100 *A. baumannii* isolates were positive for the biofilm-associated genes *bap* and *abaI*. However, the capacity to form biofilms (i.e., categorized as weak, moderate or strong) varied among the isolates with the majority of the isolates forming either moderate (n = 51/100) or weak (n = 45/100) biofilms and only a minority (n = 4/100) forming strong biofilms. Three of the four isolates forming strong biofilms were obtained from the ICU; however, no correlation was observed between the ward where the isolates were obtained and their biofilm-forming capacity. Out of 34 *A. baumannii* isolates that were obtained from the ICUs, 16 (or 47%) had moderate biofilm-forming capacity, 15 (or 44%) had weak biofilm-forming capacity and only three (or 8.8%) had strong biofilm-forming capacity. Interestingly, out of the four isolates with strong biofilm-forming ability, three of these displayed CLX MICs of > 50 µg/mL whereas the MICs for BZT and BZK ranges from 12.5 – 25 µg/mL. The remaining *A. baumannii* isolate with a strong biofilm-forming capacity, AC1642, showed a CLX MIC of 25 µg/mL (Table 1). Nevertheless, beyond these four isolates, no correlation was observed between the isolates' biofilm-forming capacity and the MIC values for the biocides. *A. baumannii* AC1605, for instance, had CLX and BZT MIC values of > 50 µg/mL but displayed only weak biofilm-forming ability (Table 1).

Discussion

There is a paucity of published data regarding biocide susceptibility in *A. baumannii*, particularly from Malaysia. To our knowledge, there has only been a single paper so far that was published on *A. baumannii* isolates obtained in 2012 – 2013 from a tertiary care teaching hospital in the capital city of Malaysia, Kuala Lumpur [13]. In that paper, high prevalence (72.95%) of *qacE* was reported among the *A. baumannii* isolates

Figure 3. The percentage of the Hospital Sultanah Nur Zahirah (HSNZ) *A. baumannii* isolates with the respective MIC values for the quaternary ammonium compounds, benzethonium chloride **(A)** and benzalkolium chloride **(B)**, according to the year of isolation.



with no *qacA* detected [13]. Although in this study, no *qacA* was also detected in the HSNZ *A. baumannii* isolates from 2011 – 2016, the prevalence of *qacE* was much lower in comparison, at 28%. The most prevalent biocide resistance gene in the HSNZ isolates was *qacΔE1* (at 63%) but Babaei *et al*. [13] did not screen for the presence of this gene in their collection of *A. baumannii* isolates. The predominance of *qacΔE1* was recently reported in carbapenem resistant *A. baumannii* isolates (96.07%) from China [11], and here the prevalence of *qacE* (at 31.37%) was similar to that of our HSNZ isolates. However, Liu *et al*. also reported the carriage of *qacA* in some of their Chinese *A. baumannii* isolates [11], albeit at a lower prevalence (13.72%), and this was similarly reported in isolates from Saudi Arabia (16.7% prevalence) [12]. This was very much in line with previous findings which showed that *qacA* is found mainly in Gram-positive bacteria as compared to Gram-negatives [14]. The Saudi study also reported similar prevalence for *qacE* (i.e., at 33.3%) in their 24 *A. baumannii* isolates that were obtained in 2015, but like Babaei *et al*. [13], the researchers did not screen for the presence of *qacΔE1* [12].

Currently, there are no standard breakpoints for biocides such as those for antibiotics in *A. baumannii* and many other pathogens. There are also differences in

the methods used for obtaining the MIC values for the biocidal agents. Here, we tried to utilize the same method (broth microdilution) used by the other Malaysian study [13] such that there will be a basis for data comparison. However, we had to modify the concentrations of biocides used in this study as we consistently obtained much higher MIC values for all the biocides tested. Babaei *et al*. reported CLX MIC values that ranged from 0.2 – 0.6 µg/mL for their *qacE*-positive isolates and even lower MIC values of 0.04 – 0.3 µg/mL for *qacE*-negative isolates [13]. In contrast, the lowest MIC value for CLX that was obtained in this study was 6.25 µg/mL with majority of the HSNZ *A. baumannii* isolates (43%) showing CLX MIC values of > 50 µg/mL. In comparing with other studies that utilized a similar broth microdilution method to determine the MIC values, Liu *et al*. reported MIC values for CLX that ranged from 4 – 64 µg/mL for the Chinese *A. baumannii* isolates [11] whereas Vijayakumar *et al*. reported MIC values of between 16 – 32 µg/mL for the Saudi isolates [12]. A study of 49 *A. baumannii* isolates from Spain yielded MIC values that ranged from 2.4 – 39.1 µg/mL for CLX [26]. Likewise, for the quaternary ammonium compounds BZT and BZK, our HSNZ *A. baumannii* isolates showed much higher MIC values than earlier reported by Babaei *et al*. [13] but the BZK values were more similar to the MIC ranges reported by Liu *et al*. [11], Vijayakumar *et al.* [12] and Fernández-Cuenca *et al*. [26] (these studies did not report on BZT). These variations reaffirm the pressing need for standardized testing methodologies as well as breakpoint values for biocides.

Our study also corroborates other reports which indicated no correlation between the carriage of biocide resistance genes such as *qacE* and *qacΔE1* with reduced biocide susceptibilities [11–13]. Nevertheless, Liu *et al*. reported that in their carbapenem-resistant *A. baumannii* strains that harboured *qacE*, a higher MIC for BZK (64 µg/mL) was observed [11], but this was not the case for our HSNZ isolates and also for the isolates from Saudi Arabia [12]. It should be noted that not all the HSNZ *A. baumannii* isolates used in this study were resistant to carbapenems. Isolates such as AC1608 and AC1621 which were carbapenem-susceptible and negative for *qacE* showed high MIC for BZK (> 50 µg/mL) whereas isolates such as AB1201 which was carbapenem-resistant and positive for *qacE* had a much lower MIC for BZK (3.125 µg/mL) (Suppplementary Table 1). Clearly further research needs to be carried out to determine the genetic basis for reduced biocide susceptibility in *A. baumannii* as the carriage of some of these genes did not appear to be a significant factor for the development of biocide resistance.

Large variations in the biofilm-forming capacity of clinical *A. baumannii* isolates have been previously reported [25,27,28] and the results of this study corroborated this. Various gene loci have been implicated in the formation of biofilms in *A. baumannii* (see review by Longo *et al*. [29]). In this study, we examined the carriage of two of these genetic determinants, *bap* and *abaI*, and found that all 100 isolates that we screened by PCR harboured these two genes. However, it should be noted that the PCR primers only detect the conserved part of these genes and whether these genes are intact and fully functional in the isolates screened is not known. This is particular pertinent for *bap* as the gene is a large (approximately 16 kb in size), repetitive locus with variations in both gene size as well as the molecular mass of the Bap protein produced [30]. Inconsistencies in *bap* gene prevalence as determined by PCR and the expression of the Bap protein have been reported [30]. Although biofilm is recognized as a contributing factor to the pathogenicity of *A. baumannii* and its capacity to persist in the harsh healthcare environment, a recent study showed that epidemic or outbreak *A. baumannii* isolates had significantly lower biofilm forming capacity when compared to sporadic isolates [28]. This led the authors to conclude that biofilm formation may not be an important factor for the epidemic spread of *A. baumannii* [28]. Interestingly, we found that three of the four strong biofilm-producing isolates from HSNZ showed high MIC values for the biocides CLX, BZT and BZK. However, no conclusive correlation was found between the biofilm-forming capacity and the MIC of biocides in the other *A. baumannii* isolates. In contrast, Hu *et al*. [28] reported that multidrug resistant (MDR) *A. baumannii* clinical isolates showed lower biofilm forming capacity as compared to non-MDR isolates. They speculated that in non-MDR isolates, the capacity to form biofilms may play a more important role in their environmental persistence in hospitals thereby placing a selective evolutionary advantage for isolates that developed high biofilm-forming capacity [28]. However, our data is not in support of this as of the four strong biofilm-producing isolates, three were MDR and only one was non-MDR (Table 1). Nevertheless, further studies need to be carried out to conclusively determine if there are any correlations between the biofilm-forming capacity of *A. baumannii* isolates and their susceptibilities/resistances to antibiotics as well as biocides.

Conclusion

In conclusion, this study has shown that the Terengganu HSNZ *A. baumannii* isolates had wide variations in their MIC values for the biocides CLX, BZT and BZK, as well as their biofilm-forming capacities. The *qacΔE1* gene is the predominant biocide resistance gene in the HSNZ isolates with no *qacA* gene detected. All the *A. baumannii* clinical isolates were positive for the *bap* and *abaI* biofilm-associated genes although the biofilm-forming capacities for the isolates were varied.

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Annex – Supplementary Items

Supplementary Table 1. Detailed characteristics of the 100 *A. baumannii* isolates from Hospital Sultanah Nur Zahirah (HSNZ), Terengganu, Malaysia.

Isolate No.	Year of isolation	Ward	Source of Isolate	Carbapenem resistance		MDR status	Antiseptic MIC (μg/ml)			*qacA*	*qacE*	*qac Δ E1*	Biofilm formation		*bap*	*abaI*
				IMP	MEM		BZK	BZT	CHX				OD	Interpretation		
AC1601	2016	medical	sputum	R	R	MDR	12.5	12.5	50	negative	Negative	Positive	0.632	Moderate (0.3-1.0)	Positive	Positive
AC1602	2016	ICU	ETT/Fluid/Aspirate	R	R	MDR	12.5	12.5	50	negative	Negative	Positive	0.647	Moderate (0.3-1.0)	Positive	Positive
AC1603	2016	ICU	Blood	S	S	Non-MDR	6.25	6.25	25	negative	Negative	Negative	0.793	Moderate (0.3-1.0)	Positive	Positive
AC1604	2016	ICU	Blood	R	R	MDR	12.5	12.5	12.5	negative	Negative	Positive	0.694	Moderate (0.3-1.0)	Positive	Positive
AC1605	2016	ICU	Pus/tissue	R	R	MDR	25	50	50	negative	Negative	Positive	0.242	Weak (<0.3)	Positive	Positive
AC1606	2016	ICU	Blood	R	R	MDR	25	25	12.5	negative	Negative	Positive	0.488	Moderate (0.3-1.0)	Positive	Positive
AC1607	2016	medical	sputum	S	S	Non-MDR	25	25	25	negative	Negative	Positive	0.654	Moderate (0.3-1.0)	Positive	Positive
AC1608	2016	ICU	ETT/Fluid/Aspirate	S	S	Non-MDR	50	25	50	negative	Negative	Positive	0.363	Weak (<0.3)	Positive	Positive
AC1609	2016	medical	sputum	R	R	MDR	12.5	25	12.5	negative	Positive	Negative	0.597	Moderate (0.3-1.0)	Positive	Positive
AC1610	2016	medical	Blood	R	R	MDR	25	25	25	negative	Positive	Negative	0.582	Moderate (0.3-1.0)	Positive	Positive
AC1611	2016	medical	Pus/tissue	R	R	MDR	25	6.25	25	negative	Positive	Negative	0.626	Moderate (0.3-1.0)	Positive	Positive
AC1612	2016	ICU	ETT/Fluid/Aspirate	R	R	MDR	6.25	50	25	negative	Negative	Negative	0.108	Weak (<0.3)	Positive	Positive
AC1613	2016	ICU	Blood	R	R	MDR	3.125	25	25	negative	Negative	Negative	0.3	Weak (<0.3)	Positive	Positive
AC1614	2016	hd/hb	Pus/tissue	S	S	Non-MDR	25	25	25	negative	Negative	Negative	0.379	Moderate (0.3-1.0)	Positive	Positive
AC1615	2016	medical	sputum	R	R	MDR	3.125	6.25	6.25	negative	Negative	Negative	0.638	Moderate (0.3-1.0)	Positive	Positive
AC1616	2016	hd/hb	sputum	R	R	MDR	6.25	12.5	25	negative	Negative	Negative	0.129	Weak (<0.3)	Positive	Positive
AC1617	2016	hd/hb	Blood	S	S	Non-MDR	12.5	12.5	25	negative	Negative	Positive	0.598	Moderate (0.3-1.0)	Positive	Positive
AC1618	2016	medical	ETT/Fluid/Aspirate	R	R	MDR	50	50	25	negative	Negative	Negative	0.175	Weak (<0.3)	Positive	Positive
AC1619	2016	hd/hb	Urine	S	S	Non-MDR	25	50	50	negative	Negative	Positive	0.692	Moderate (0.3-1.0)	Positive	Positive
AC1620	2016	medical	Blood	S	S	Non-MDR	12.5	12.5	50	negative	Negative	Positive	0.72	Moderate (0.3-1.0)	Positive	Positive
AC1621	2016	medical	Blood	S	S	Non-MDR	50	12.5	25	negative	Negative	Positive	0.2066	Weak (<0.3)	Positive	Positive
AC1622	2016	medical	Blood	S	S	Non-MDR	12.5	12.5	50	negative	Negative	Negative	1.097	Strong (>1.0)	Positive	Positive
AC1623	2016	ICU	Blood	S	S	Non-MDR	25	50	12.5	negative	Negative	Positive	0.8552	Moderate (0.3-1.0)	Positive	Positive
AC1624	2016	medical	Pus/tissue	R	R	MDR	25	50	50	negative	Negative	Negative	0.387	Moderate (0.3-1.0)	Positive	Positive
AC1625	2016	Ortho	Pus/tissue	R	R	MDR	25	50	25	negative	Negative	Positive	0.7647	Moderate (0.3-1.0)	Positive	Positive
AC1626	2016	medical	Blood	R	R	MDR	12.5	6.25	25	negative	Positive	Positive	0.504	Moderate (0.3-1.0)	Positive	Positive
AC1627	2016	medical	Pus/tissue	R	R	MDR	25	25	50	negative	Negative	Positive	0.4876	Moderate (0.3-1.0)	Positive	Positive
AC1628	2016	medical	Pus/tissue	S	S	Non-MDR	50	25	12.5	negative	Positive	Positive	0.4542	Moderate (0.3-1.0)	Positive	Positive
AC1629	2016	medical	Pus/tissue	R	R	MDR	12.5	12.5	25	negative	Positive	Positive	0.2197	Weak (<0.3)	Positive	Positive
AC1630	2016	medical	Pus/tissue	R	R	MDR	6.25	50	6.25	negative	Positive	Positive	0.3181	Weak (<0.3)	Positive	Positive
AC1631	2016	hd/hb	Blood	S	S	Non-MDR	50	50	25	negative	Negative	Negative	0.678	Moderate (0.3-1.0)	Positive	Positive
AC1632	2016	medical	Blood	R	R	MDR	25	25	50	negative	Positive	Positive	0.7066	Moderate (0.3-1.0)	Positive	Positive
AC1633	2016	ICU	Blood	R	R	MDR	12.5	12.5	50	negative	Negative	Positive	0.5985	Moderate (0.3-1.0)	Positive	Positive
AC1634	2016	medical	Pus/tissue	R	R	MDR	25	25	25	negative	Positive	Positive	0.6795	Moderate (0.3-1.0)	Positive	Positive
AC1635	2016	ICU	ETT/Fluid/Aspirate	R	R	MDR	25	6.25	50	negative	Negative	Positive	0.0793	Weak (<0.3)	Positive	Positive
AC1636	2016	ICU	Pus/tissue	R	R	MDR	12.5	25	50	negative	Negative	Positive	1.069	Strong (>1.0)	Positive	Positive
AC1637	2016	ICU	sputum	S	S	Non-MDR	12.5	12.5	12.5	negative	Positive	Negative	0.3468	Weak (<0.3)	Positive	Positive
AC1638	2016	medical	Eye	S	S	Non-MDR	12.5	12.5	25	negative	Negative	Negative	0.2491	Weak (<0.3)	Positive	Positive
AC1639	2016	medical	Urine	S	S	Non-MDR	12.5	12.5	50	negative	Negative	Positive	0.557	Moderate (0.3-1.0)	Positive	Positive
AC1640	2016	medical	ETT/Fluid/Aspirate	S	S	Non-MDR	12.5	12.5	50	negative	Negative	Negative	0.2857	Weak (<0.3)	Positive	Positive
AC1641	2016	ICU	Eye	S	S	Non-MDR	12.5	12.5	25	negative	Positive	Positive	0.7435	Moderate (0.3-1.0)	Positive	Positive
AC1642	2016	ICU	ETT/Fluid/Aspirate	R	R	MDR	25	25	25	negative	Negative	Positive	1.0409	Strong (>1.0)	Positive	Positive
AC1643	2016	hd/hb	ETT/Fluid/Aspirate	R	R	MDR	12.5	25	50	negative	Negative	Positive	0.8019	Moderate (0.3-1.0)	Positive	Positive
AC1644	2016	ICU	Urine	S	S	Non-MDR	12.5	25	25	negative	Negative	Positive	0.7021	Moderate (0.3-1.0)	Positive	Positive
AC1645	2016	medical	Blood	R	R	MDR	12.5	25	50	negative	Positive	Positive	0.068	Weak (<0.3)	Positive	Positive
AC1646	2016	Ortho	sputum	S	S	Non-MDR	12.5	25	50	negative	Positive	Positive	0.5747	Moderate (0.3-1.0)	Positive	Positive
AC1647	2016	medical	Blood	S	S	Non-MDR	12.5	6.25	12.5	negative	Negative	Negative	0.8612	Moderate (0.3-1.0)	Positive	Positive
AC1648	2016	ICU	Blood	S	S	Non-MDR	12.5	12.5	6.25	negative	Negative	Negative	0.7551	Moderate (0.3-1.0)	Positive	Positive
AC1649	2016	medical	sputum	S	S	Non-MDR	12.5	25	50	negative	Negative	Negative	0.1543	Weak (<0.3)	Positive	Positive
AC1650	2016	scn	Eye	S	S	Non-MDR	12.5	25	12.5	negative	Positive	Positive	0.1032	Weak (<0.3)	Positive	Positive
AC1501	2015	ICU	ETT/Fluid/Aspirate	R	R	MDR	6.25	12.5	50	negative	Negative	Positive	0.2949	Weak (<0.3)	Positive	Positive
AC1502	2015	ICU	ETT/Fluid/Aspirate	S	S	Non-MDR	6.25	6.25	25	negative	Negative	Negative	0.4712	Moderate (0.3-1.0)	Positive	Positive
AC1503	2015	medical	sputum	R	R	MDR	12.5	6.25	25	negative	Negative	Negative	0.1221	Weak (<0.3)	Positive	Positive
AC1504	2015	medical	sputum	S	S	Non-MDR	25	25	50	negative	Negative	Positive	0.2839	Weak (<0.3)	Positive	Positive
AC1505	2015	Ortho	Pus/tissue	R	R	MDR	6.25	6.25	12.5	negative	Negative	Positive	0.156	Weak (<0.3)	Positive	Positive
AC1507	2015	medical	Blood	S	S	Non-MDR	12.5	25	25	negative	Negative	Positive	0.2988	Weak (<0.3)	Positive	Positive
AC1508	2015	medical	sputum	S	S	Non-MDR	6.25	12.5	12.5	negative	Negative	Negative	0.0865	Weak (<0.3)	Positive	Positive
AC1509	2015	medical	sputum	S	S	Non-MDR	6.25	6.25	12.5	negative	Negative	Negative	0.153	Weak (<0.3)	Positive	Positive
AC1510	2015	medical	sputum	S	S	Non-MDR	12.5	12.5	12.5	negative	Negative	Negative	0.2591	Weak (<0.3)	Positive	Positive
AC1511	2015	medical	sputum	S	S	Non-MDR	12.5	25	25	negative	Negative	Negative	0.4842	Moderate (0.3-1.0)	Positive	Positive
AC1512	2015	hd/hb	sputum	R	R	MDR	6.25	25	12.5	negative	Negative	Positive	0.1148	Weak (<0.3)	Positive	Positive
AC1513	2015	medical	sputum	R	R	MDR	6.25	6.25	25	negative	Negative	Positive	0.0605	Weak (<0.3)	Positive	Positive
AC1514	2015	ICU	sputum	R	R	MDR	3.125	6.25	25	negative	Negative	Positive	0.0839	Weak (<0.3)	Positive	Positive
AC1515	2015	medical	ETT/Fluid/Aspirate	R	R	MDR	3.125	6.25	12.5	negative	Negative	Negative	0.1013	Weak (<0.3)	Positive	Positive
AC1516	2015	PICU	ETT/Fluid/Aspirate	S	S	Non-MDR	12.5	6.25	50	negative	Negative	Negative	0.0953	Weak (<0.3)	Positive	Positive

AC1517	2015	medical	Blood	S	S	Non-MDR	12.5	25	50	negative	Negative	Positive	0.2047	Weak (<0.3)	Positive	Positive
AC1518	2015	medical	Blood	R	R	MDR	12.5	25	50	negative	Negative	Positive	0.2848	Weak (<0.3)	Positive	Positive
AC1519	2015	PICU	ETT/Fluid/Aspirate	R	R	MDR	6.25	12.5	50	negative	Negative	Positive	0.2507	Weak (<0.3)	Positive	Positive
AC1523	2015	medical	Urine	R	R	MDR	12.5	12.5	25	negative	Negative	Positive	0.077	Weak (<0.3)	Positive	Positive
AC1524	2015	medical	Pus/tissue	R	R	MDR	12.5	12.5	50	negative	Negative	Negative	0.081	Weak (<0.3)	Positive	Positive
AC1525	2015	medical	Blood	S	S	Non-MDR	6.25	12.5	12.5	negative	Negative	Positive	0.0431	Weak (<0.3)	Positive	Positive
AC1526	2015	medical	Blood	R	R	MDR	12.5	12.5	50	negative	Negative	Positive	0.1045	Weak (<0.3)	Positive	Positive
AC1527	2015	medical	sputum	R	R	MDR	12.5	25	50	negative	Negative	Negative	0.1298	Weak (<0.3)	Positive	Positive
AC1528	2015	ICU	ETT/Fluid/Aspirate	R	R	MDR	12.5	25	6.25	negative	Negative	Positive	0.057	Weak (<0.3)	Positive	Positive
AC1529	2015	ENT	Blood	S	S	Non-MDR	6.25	12.5	50	negative	Negative	Negative	0.1919	Weak (<0.3)	Positive	Positive
AC1530	2015	medical	Blood	R	R	MDR	25	25	25	negative	Negative	Negative	0.3967	Moderate (0.3-1.0)	Positive	Positive
AC1532	2015	ICU	Blood	R	R	MDR	1.56	6.25	12.5	negative	Negative	Positive	0.3547	Moderate (0.3-1.0)	Positive	Positive
AC1535	2015	medical	ETT/Fluid/Aspirate	R	R	MDR	12.5	12.5	50	negative	Negative	Positive	0.403	Moderate (0.3-1.0)	Positive	Positive
AC1536	2015	ICU	Blood	S	S	Non-MDR	3.125	12.5	12.5	negative	Negative	Positive	0.1046	Weak (<0.3)	Positive	Positive
AC1537	2015	medical	Blood	S	S	Non-MDR	1.56	25	50	negative	Negative	Negative	0.0981	Weak (<0.3)	Positive	Positive
AB1201	2012	ICU	Pus/tissue	R	R	MDR	3.125	25	50	negative	Positive	Positive	0.246	Weak (<0.3)	Positive	Positive
AB1202	2012	ICU	Urine	R	R	MDR	12.5	12.5	50	negative	Positive	Positive	1.16	Strong (>1.0)	Positive	Positive
AB1204	2012	ICU	Pus/tissue	R	R	MDR	25	25	12.5	negative	Positive	Positive	0.7	Moderate (0.3-1.0)	Positive	Positive
AB1205	2012	Ortho	Pus/tissue	R	R	MDR	12.5	25	50	negative	Positive	Positive	0.285	Weak (<0.3)	Positive	Positive
AB1206	2012	Ortho	Pus/tissue	R	R	MDR	12.5	25	50	negative	Positive	Positive	0.236	Weak (<0.3)	Positive	Positive
AB1208	2012	ICU	Blood	R	R	MDR	12.5	6.25	50	negative	Positive	Positive	0.315	Weak (<0.3)	Positive	Positive
AB1213	2012	ICU	ETT/Fluid/Aspirate	R	R	MDR	25	12.5	12.5	negative	Positive	Positive	0.644	Moderate (0.3-1.0)	Positive	Positive
AB1217	2012	ENT	Blood	S	S	Non-MDR	6.25	6.25	12.5	negative	Positive	Positive	0.743	Moderate (0.3-1.0)	Positive	Positive
AB1218	2012	ICU	Blood	R	R	MDR	12.5	12.5	25	negative	Negative	Negative	0.206	Weak (<0.3)	Positive	Positive
AB1220	2012	Ortho	Pus/tissue	R	R	MDR	25	12.5	50	negative	Positive	Positive	0.164	Weak (<0.3)	Positive	Positive
AB1221	2012	Ortho	Blood	R	R	MDR	12.5	6.25	25	negative	Positive	Positive	0.205	Weak (<0.3)	Positive	Positive
AB1222	2012	Ortho	Pus/tissue	R	R	MDR	12.5	12.5	25	negative	Positive	Positive	0.421	Moderate (0.3-1.0)	Positive	Positive
AB1223	2012	ICU	Blood	R	R	MDR	12.5	3.125	50	negative	Positive	Positive	0.156	Weak (<0.3)	Positive	Positive
AB1224	2012	Haemodialysis	Pus/tissue	R	R	Non-MDR	12.5	12.5	50	negative	Positive	Positive	0.199	Weak (<0.3)	Positive	Positive
AC1106	2011	Ortho	Pus/tissue	R	R	MDR	25	25	50	negative	Negative	Positive	0.3773	Moderate (0.3-1.0)	Positive	Positive
AC1108	2011	Haemodialysis	ETT/Fluid/Aspirate	R	R	MDR	12.5	6.25	50	negative	Negative	Negative	0.2596	Weak (<0.3)	Positive	Positive
AC1119	2011	ICU	ETT/Fluid/Aspirate	R	R	MDR	12.5	6.25	25	negative	Negative	Negative	0.4298	Moderate (0.3-1.0)	Positive	Positive
AC1123	2011	PICU	ETT/Fluid/Aspirate	R	R	MDR	12.5	12.5	25	negative	Positive	Negative	0.4157	Moderate (0.3-1.0)	Positive	Positive
AC1138	2011	medical	ETT/Fluid/Aspirate	R	R	MDR	12.5	12.5	50	negative	Negative	Positive	0.666	Moderate (0.3-1.0)	Positive	Positive
AC1140	2011	ICU	ETT/Fluid/Aspirate	R	R	MDR	6.25	12.5	25	negative	Negative	Negative	0.5655	Moderate (0.3-1.0)	Positive	Positive

Supplementary Table 2. Sequences of primers and PCR amplification conditions used in this study.

Genes	Primer	Primer sequence (5' → 3')	Expected size (bp)	PCR conditions	Reference
qacE	Forward	ATGAAAGGCTGGCTT	300	Pre-denaturation: 94ºC for 3 minutes, 35 cycles of denaturation: 94ºC for 45 s; annealing: 55ºC for 45 seconds, and extension: 72ºC for 45 s. Final extension: 72ºC for 8 minutes	[13]
	Reverse	TCACCATGGCGTCGG			
qacΔE1	Forward	TAGCGAGGGCTTTACTAAGC	335	Pre-denaturation: 93ºC for 2 minutes, 35 cycles of denaturation: 94ºC for 30 seconds; annealing: 55ºC for 30 seconds; and extension: 72ºC for 1 minute. Final extension: 72ºC for 5 minutes.	[31]
	Reverse	ATTCGAAATGCCGAACACCG			
bap	Forward	TACTTCCAATCCAATGCTAGGGAGGGTACCAATGCAG	1500	Pre-denaturation: 95ºC for 3 minutes, 30 cycles of denaturation: 95ºC for 1 minute; annealing: 56ºC for 1 minute; and extension: 72ºC for 1 minute. Final extension: 72ºC for 5 minutes.	[32]
	Reverse	TTATCCACTTCCAATGATCAGCAACCAAACCGCTAC			
abaI	Forward	GTACAGTCGACGTATTTGTTGAATATTTGGG	382	Pre-denaturation: 94ºC for 10 minutes, 30 cycles of denaturation: 94ºC for 30 seconds; annealing: 66.5ºC for 30 seconds; and extension: 72ºC for 1 minute. Final extension: 72ºC for 5 minutes.	[20]
	Reverse	CGTACGTCTAGAGTAATGAGTTGTTTTGCGCC			