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Unravelling Virulence Activities of Hospital Isolated *Acinetobacter baumannii*: Exploring the Prospective Application of Aspirin as an Antivirulence Agent

(Membongkar Aktiviti Kevirulenan *Acinetobacter baumannii* Pencilan Hospital: Meneroka Prospektif Penggunaan Aspirin sebagai Agen Antivirulen)

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ABSTRACT

Acinetobacter baumannii infections pose a global public health threat due to the increasing resistance to various antimicrobial agents. This study reports the virulence characteristics of *A. baumannii* strains isolated from patients at Hospital Canselor Tuanku Muhriz UKM (HCTM) and explores the potential of aspirin as an antivirulence agent. Fourteen *A. baumannii* isolates from various infection sites exhibited resistance to at least two antibiotics. Among them, 43% (n=6) displayed high motility, correlating with the site of isolation. Additionally, 58% (n=7) formed strong biofilms, 36% (n=5) secreted proteases and 36% (n=5) resisted oxidative stress. Notably, isolates Ab_H4 and Ab_H10 displayed the highest virulence, warranting further investigation. Molecular analysis using polymerase chain reaction (PCR) showed that both Ab_H4 and Ab_H10 possessed the *bap* and *katG* genes. Although both strains were capable of secreting proteases, only Ab_H4 possessed the *cpaA* gene, suggesting the involvement of other genes in protease secretion in Ab_H10. Despite high motility, no *pilT* gene was detected in any isolates. Treatment with sub-inhibitory concentrations of aspirin (3.25 mg/ml) restored susceptibility to previously resistant antibiotics, disrupted biofilm formation, and reduced proteases and catalases secretion. However, no significant impact on bacterial motility was observed. Interestingly, sub-inhibitory concentrations of aspirin induced biofilm formation in the environmental strain control (Ab_UKMCC), which was significantly reduced upon exposure to the MIC. These findings highlight the high virulence capabilities of several strains isolated from HCTM and show the potential of aspirin as an antivirulence agent, offering valuable insights for combating *A. baumannii* infections.

Keywords: *Acinetobacter baumannii*; antivirulence; aspirin; biofilm; virulence

ABSTRAK

Jangkitan *Acinetobacter baumannii* adalah ancaman kesihatan awam global kerana peningkatan kerintangan terhadap pelbagai agen antimikrob. Kajian ini melaporkan ciri virulen *A. baumannii* yang dipencilkan daripada pesakit di Hospital Canselor Tuanku Muhriz UKM (HCTM) dan meneroka potensi aspirin sebagai agen antivirulen. Empat belas strain *A. baumannii* daripada pelbagai tapak jangkitan menunjukkan kerintangan terhadap sekurang-kurangnya dua antibiotik. Sebanyak 43% (n=6) pencilan menunjukkan tahap motiliti yang tinggi berkolerasi dengan lokasi pencilan. Selain itu, 58% (n=7) membentuk biofilem yang kuat, 36% (n=5) merembeskan protease dan 36% (n=5) menahan tekanan oksidatif. Pencilan Ab_H4 dan Ab_H10 menunjukkan kevirulenan tertinggi yang memerlukan penyelidikan lanjutan. Analisis molekul menggunakan tindak balas berantai polimerase (PCR) menunjukkan kedua-dua Ab_H4 dan Ab_H10 mempunyai gen *bap* dan *katG*. Walaupun kedua-dua strain mampu menghasilkan protease, hanya Ab_H4 memiliki gen *cpaA*, mencadangkan keterlibatan gen lain dalam rembesan protease di Ab_H10. Walaupun mempunyai motiliti tinggi, tiada gen *pilT* dikesan dalam mana-mana pencilan. Rawatan dengan kepekatan sub-perencatan minimum aspirin (3.25 mg/mL) mengembalikan kerentanan terhadap antibiotik yang sebelumnya adalah rintang, mengurangkan pembentukan biofilem, pengeluaran protease dan katalase tetapi tiada kesan yang signifikan dalam motiliti bakteria. Tambahan lagi, kepekatan sub-perencatan minimum aspirin merangsang pembentukan biofilem dalam strain pencilan persekitaran (Ab_UKMCC), namun pengurangan ketara diperhatikan apabila terdedah kepada kepekatan minimum perencatan (MIC) aspirin. Penemuan ini menunjukkan keupayaan virulen yang tinggi bagi beberapa strain *A. baumannii* pencilan HCTM serta menunjukkan potensi

aspirin sebagai agen antivirulen, menawarkan sumbangan bernilai dalam usaha berterusan untuk memerangi jangkitan *A. baumannii*.

Kata kunci: *Acinetobacter baumannii*; antivirulens; aspirin; biofilem; virulen

INTRODUCTION

Bacterial infections remain one of the worrying global burdens that contributes to the high morbidity and mortality around the world, especially in developing countries (Kraus 2008). The excessive prescription of antibiotics on top of poor preventative measures drives the surge of antibiotic resistance bacteria, which further exacerbates the problem (English & Gaur 2010). In 2019, the Centre for Disease Control (CDC) published a staggering number of 2.8 million antibiotic-resistant infection cases reported annually in the US, which resulted in more than 35,000 deaths. In this report, several bacterial species have been identified with significant healthcare burden, including carbapanem-resistant *Acinetobacter* bacteria, which is listed as an urgent threat that causes over 8,000 cases annually in the US alone.

Acinetobacter bacteria frequently contaminate medical equipment and is one of the major attributes for nosocomial infections (Babaei et al. 2015; Peleg, Seifert & Paterson 2008). Among this bacteria group, *Acinetobacter baumannii* is the most significant strain being associated with infections in human and clinical settings. Infections caused by the carbapanem- resistant strain of this bacterial species have been worryingly high in Southeast Asia countries like Malaysia, Singapore, and the Philippines, and the highest cases were reported in Thailand (Janahiraman et al. 2015; Rani et al. 2017). Recent pandemics also witnessed higher *A. baumannii* infections among COVID-19 patients, especially in the Intensive Care Unit (ICU) which further worsened the morbidity (Russo et al. 2022).

Bacterial infections caused by *A. baumannii* led to various complications ranging from wound infection to severe diseases, such as bacteraemia, pneumonia and meningitis (Vázquez-López et al. 2020). These complications were attributed to the virulence capabilities of *A. baumannii*, such as its motility, production of exotoxins, protease and phospholipase secretion, as well as catalase production, which led to oxidative resistance during infection (Moubareck & Halat 2020; Wong et al. 2017). Several *A. baumannii* strains are also capable of forming robust biofilm, which aids in its persistence in hospital settings, thus making the disinfection process a strenuous labour (Chapartegui-González et al. 2018). Hence, there is a vital need in assessing the virulence of each bacterial strain to monitor the occurrence of virulence gene transfer as well as to draft a better treatment plan.

Several antivirulence agents have been studied to successfully target multiple virulence factors of *A. baumannii*, including LpxC-1, deferiprone and virstatin (García-Quintanilla et al. 2016; Nait Chabane et al. 2014; Thompson et al. 2012). Recently, more research was further conducted in exploring other antivirulence agents that could be used to inhibit virulence action in *A. baumannii*. Among the potential antivirulence agents that are being studied are silver nanoparticles (Hetta et al. 2021), pyrogallol (Abirami et al. 2021), flavonoids and curcumin (Raorane et al. 2019), myrtenol (Selvaraj et al. 2020), as well as rhamnolipids (Firdose et al. 2024). However, all research remains at an early investigational stage. Seleem et al. (2020) studied the antivirulence properties of routine drugs, such as erythromycin, levamisole, choloroquine, and propranolol, and found great promise in their virulence's inhibitory activity. Aspirin has long been recognised as a prominent antipyretic, and its impact on the antibiotic susceptibility of bacteria was demonstrated in Zimmermann and Curtis (2018). Beyond influencing antibiotic susceptibility, its potential as an antibiotic was also comprehensively reviewed in Zimmermann and Curtis (2017). Recognising this potential, aspirin could be repurposed as an antivirulence, offering a promising avenue for more effective treatment of *A. baumannii* infections, particularly those originating from nosocomial sources. The ability of aspirin as a quorum quencher that successfully reduces virulence characteristics has been proven in *Pseudomonas aeruginosa* and was confirmed with stochastic analysis using molecular modelling (El-Mowafy et al. 2014). However, its effect on *A. baumannii* was never studied.

Therefore, the primary objective of this research was to analyse the virulence characteristics exhibited by *A. baumannii* strains isolated from patients receiving treatment at Tuanku Muhriz UKM Chancellor's Hospital. The investigation concentrated on evaluating the strains' motility, protease and catalase production, as well as their capacity for biofilm formation, a crucial factor influencing their pathogenic nature. The study also explored the presence of genes linked to virulence, encompassing *bap*, *pilT*, *cpaA* and *katG* genes, to establish their correlation with these virulence manifestations. Additionally, this research delved into exploring the potential of aspirin in diminishing both virulence and biofilm activity in *A. baumannii*.

MATERIALS AND METHODS

BACTERIAL STRAINS AND CULTURE CONDITIONS

A total of 12 *A. baumanni* cultures were collected from the Bacteriology Unit, Department of Diagnostic Laboratory Services, Tuanku Muhriz UKM Chancellor's Hospital (HCTM). Bacterial cultures were isolated from several invasive and non-invasive sites, primarily from blood and tracheal aspirate. Two strains were obtained a year

prior from the same hospital and stored in the university laboratory storage. The isolation site for the strains, however, was unknown. Table 1 describes the isolation location for each bacterial strain. *A. baumanni* obtained from university culture collection, Ab_UKMCC, which was isolated from the environment, was used as a reference strain. All bacterial isolates were grown overnight at 37 °C in lysogeny broth (LB) and incubated with agitation at 215 rpm.

ANTIBIOGRAM

The antibiogram of all isolated strains was conducted using the Kirby Bauer disc diffusion method (Biemer 1973). The antibiotics used were 10 µg Ampicillin, 15 µg Erythromycin, 30 µg Cefazolin, 5 µg Ciprofloxacin, 10 µg Gentamycin, 300 µg Polymyxin B, 30 µg Tetracycline, 30 µg Chloramphenicol and 10 µg Imipenem. One disc containing saline water was placed as a control. The plates were then incubated in an inverted position at 37 °C and the diameter of the zone of inhibitions was calculated after 18 h. The diameter of inhibition zones was measured, and the susceptibility of the bacteria was interpreted according to the Clinical Laboratory Standards Institute (CLSI) (M100-ED33:2023) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) (v13.0:2023).

The capability of *A. baumannii* isolates to form biofilm was tested with 96-wells titre plate. A colony was collected and suspended in 10 mL of Luria Bertani broth (LB broth). The culture was then adjusted to 5×10^5 CFU/mL before 100 µL of aliquots were transferred into 96-well plates and incubated at 37 °C for 48 h. After 48 h, the bacterial colonies were washed with Phosphate-buffered saline (PBS) (10 mM Na_2PO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.0) to remove all free planktonic cells. The plate was then airdried before 200 µm of 0.5% crystal violet solution was added to the air-dried biofilm for another 15 min prior to washing with PBS solution for three times to remove excess stain. The biofilms formed were dissolved in 95% ethanol and quantified via absorbance value at OD of 595 nm using a microplate reader. The experiment was conducted in triplicate, and the average optical densities were determined. For the negative control, wells containing only LB media were used.

To analyse the biofilm-forming properties of these strains, the criteria employed by Stepanović et al. (2007) was used. Briefly, the cut-off value (ODc) was first determined using the formula ODc = mean OD of negative control + (3 × SD of negative control). For each tested strain, the final OD value is obtained by subtracting the established ODc value from the average OD value of the respective strain. In instances where a negative value arises because of these calculations, it is represented as zero, signifying absence of biofilm production. The strains' biofilm-forming capabilities are systematically classified into distinct groups, delineated by their optical density (OD) values as follows: The non-biofilm producers ($OD \leq ODc$); weak biofilm producers ($ODc < OD \leq 2 \times ODc$); moderate biofilm producers ($2 \times ODc < OD \leq 4 \times ODc$); and strong biofilm producers ($4 \times ODc < OD$).

TABLE 1. Details of bacterial isolates collected from Bacteriology Unit, Department of Diagnostic Laboratory Services, Tuanku Muhriz UKM Chancellor's Hospital

Isolate No	Isolation site
Ab_H1	Tracheal Aspirate
Ab_H2	Blood
Ab_H3	Blood
Ab_H4	Tracheal Aspirate
Ab_H5	Blood
Ab_H6	Tracheal Aspirate
Ab_H7	Blood
Ab_H8	Tracheal Aspirate
Ab_H9	Pus
Ab_H10	Blood
Ab_H11	Tracheal Aspirate
Ab_H12	Tracheal Aspirate
Ab_ESKAPE 1	Unknown
Ab_ESKAPE 2	Unknown

BIOFILM ANALYSIS

MOTILITY ASSAY

The motility of the isolates on the surface was determined using the procedure described in Cosgaya et al. (2019). Briefly, semisolid LB media was prepared by supplementing LB broth (tryptone 10 g/L, NaCl 1.25 g/L, yeast extract 5 g/L) with 0.8% agar. All plates were freshly prepared on the day of inoculation. 15 mL of the medium was poured into a petri dish and was allowed to air dry with a closed lid in a laminar flow hood for 20 min. The bacterial colony was picked up and diluted in 1 mL of LB broth before being adjusted to 0.5 McFarland. 2 µL of the cultures were stab-inoculated into the middle layer of semisolid agar, respectively. The plate was then incubated for 48 h at 37 °C. Swarming positive isolates were confirmed by measuring zones around the site of inoculation that have more than 10 mm diameter.

The average diameter of the migration zone, obtained from three readings, was then utilised to classify the motility of the isolates based on the criteria outlined in Vijayakumar et al. (2016). Non-motile isolates were characterised by a zone of travel ≤ 1.0 cm; those with intermediate motility exhibited a zone of travel between 1.0-1.5 cm; and highly motile isolates displayed a zone of travel ≥ 1.5 cm.

SCREENING FOR PROTEASE PRODUCTION

Protease activity was performed on skimmed milk agar (SMA), which was prepared using a 10% (w/v) stock solution of skimmed milk powder autoclaved at 115 °C for 10 min, while the agar solution was made separately and autoclaved at 121 °C for 20 min. The two solutions were mixed to a final concentration of 1% skimmed milk while still hot and poured into Petri plates. The tested strain was spotted on skimmed milk agar plates and incubated for 3 days at 37 °C. The formation of a clear zone around the colony was considered protease-positive. The isolates that are capable of producing protease were identified and their protease production is quantified.

PROTEASE QUANTIFICATION

A. baumannii strain that showed protease production was subjected to protease quantification using azocasein-degrading proteolytic activity. The protocol was conducted as described in Junior et al. (2020), with some modifications to the volume used. Briefly, a single colony of *A. baumannii* isolate, which was previously identified as a protease producer, was inoculated overnight in brain-heart infusion broth at 37 °C. 0.5 mL of bacteria suspension was then inoculated in nutrient broth (NB) and incubated overnight in a shaker incubator at 200 rpm, 37 °C. 100 µL of *A. baumannii* culture supernatant was added into 1 mL of 3 mg azocasein prepared in 0.05 M Tris HCl and 0.5 mM $CaCl_2$ adjusted to pH 7.5 before being incubated at 37 °C for 1 h. The reaction was terminated by the addition of 0.5 mL of 10% trichloroacetic acid (TCA), and the tube was incubated on ice for 30 min. The precipitated protein was removed by centrifugation at 10,000 × g for 20 min. 1 mL of 1 M NaOH was added in an equal ratio of supernatant and mixed well. The enzyme activity was estimated based on the degradation of casein into smaller peptides and soluble amino acids. The absorbance of the supernatant was then determined at 440 nm. The activity was expressed in relative enzymatic units (U), where 1 U is one unit of protease that causes an increase of 0.01 per min in the absorbance measured at A440 nm. Triplicate readings were taken, and the mean value of these readings was used to construct the graph.

CATALASE ASSAY AND QUANTIFICATION

The aliquots of bacterial cultures (100 µL) grown in LB broth were obtained during its stationary growth phase and spread on MH agar plates. Sterile 6 mm diameter paper discs were placed evenly on the surface of the agar and spotted with 10 µL of 1.5% hydrogen peroxide. The plates were incubated overnight at 37 °C, and the diameter of growth inhibition around the disc was measured. The clear zone surrounding the disc indicates bacterial death due to oxidative stress exerted by the hydrogen peroxide, and the smaller clear zone diameter indicates higher resistance towards oxidative stress mediated by catalase production.

The steps for quantifying catalase activity were carried out as follows, as described in Bhargava, Sharma and Capalash (2014). The reaction mixture was prepared by combining 500 µL of 5 mM hydrogen peroxide (H_2O_2) with 400 µL of 50 mM phosphate buffer (pH 7.0). The resulting mixture served as the reaction medium. Subsequently, 100 µL of the crude enzyme, containing the catalase enzyme, was added to the reaction mixture. The reaction mixture was then placed in a spectrophotometer, and the wavelength was set to 240 nm. Absorbance measurements were recorded at 10 s intervals for a duration of 5 min. To establish a control, a separate sample was prepared by substituting the crude enzyme with H_2O_2-free phosphate buffer. Catalase activity was calculated by comparing the rate of H_2O_2 decomposition in the reaction mixture to that of the control sample. One unit of catalase activity was defined as the amount of enzyme that decomposed 1 mM of H_2O_2 per minute at room temperature. Finally, the catalase activity was expressed in units per milligram (U/mg) of protein by dividing the catalase activity by the amount of protein in milligrams used in the assay.

PRESENCE OF VIRULENCE-ASSOCIATED GENES

A. baumannii strain was grown overnight, and its genomic DNA was extracted using the ABCAM DNA (ab156900) extraction kit following the manufacturer's instructions. Amplification of the gene of interest was conducted in a Bio-Rad MyCycler thermal cycler (Bio-Rad, USA) before the presence of the gene was visualised using gel electrophoresis. All genes were amplified under the following conditions: 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, annealing temperature for 30 s, 72 °C for 2 min and a final step of 72 °C for 10 min. The primer sequence and annealing temperature for each primer used in this study were listed in Table 2. Electrophoresis was then conducted with 1.5% agarose gel to separate the products. The presence of virulence-associated genes in the bacterial genomics was visualised under a UV transilluminator based on the amplicon size at the visualised bands.

DETERMINATION OF MIC OF ASPIRIN

The inoculation procedure with aspirin was performed following the method described by El-Mowafy et al. (2014) with some modifications. Initially, 400 mg of aspirin was diluted in 1 mL of LB broth and subsequent 2-fold serial dilutions were conducted to obtain concentrations of 400, 200, 100, 50, 25, 12.5, 6 and 3 mg/mL. A lawn of *A. baumannii* was prepared on LB agar, and then 20 µL of the diluted aspirin solution was applied to a paper disc. The disc was allowed to air dry under sterile conditions before being placed on the bacterial lawn. The minimum inhibitory concentration (MIC) of aspirin was determined as the lowest concentration that inhibited visible bacterial growth around the disc.

ANTIMICROBIAL SUSCEPTIBILITY ASSAY

Susceptibility assays were conducted using the disc diffusion method, as described in the antibiogram analysis. The bacterial strains were cultured overnight in LB broth, supplemented with 1/2 of the minimum inhibitory concentration (MIC) of aspirin, under shaking conditions (37 °C, 200 rpm). Subsequently, the strains were tested against the same antibiotic discs used in the previous antibiogram analysis.

BIOFILM DISRUPTION ASSAY

A single colony was selected and inoculated into 5 mL of LB broth. The optical density at 595 nm (OD595) was measured and adjusted to 1.0 McFarland standard. Next, 200 µL of the bacterial culture was pipetted into the wells of a 96-well titre plate. To assess the effect of aspirin, 50 µL of a 1/4 minimum inhibitory concentration (MIC) and 1/2 MIC aspirin solution were added to separate wells. The control well contained 50 µL of LB broth instead of the aspirin solution. The plate was then incubated at 37 °C for 48 h, allowing biofilm formation. Subsequently, the biofilm was quantified using the previously mentioned method.

MOTILITY INHIBITION ASSAY

The bacterial strains were initially cultured overnight in LB broth, supplemented with 1/2 of the minimum inhibitory concentration (MIC) of aspirin, under continuous agitation (37 °C, 200 rpm). After aspirin treatment, the motility assay was replicated using the same methods as previously described. The extent of travel zones was measured and compared with the pre-treatment values. These readings were captured in triplicate and any variations in motility were calculated to determine if significant changes occurred.

ENZYME SECRETION POST INHIBITION ASSAY

The bacterial strains were cultured overnight in LB broth, supplemented with 1/2 of the minimum inhibitory concentration (MIC) of aspirin, under continuous agitation (37 °C, 200 rpm). The bacterial culture was then centrifuged (10,000 rpm, 10 min) to obtain the cell-free culture supernatant. The supernatant was then subjected to protease and catalase quantification like the procedure previously mentioned.

SCANNING ELECTRON MICROSCOPY

For the preparation of the sample for SEM, critical point dryer was used before being analysed using Variable Pressure Scanning Electron Microscope (VPSEM) located at Electron Microscopy Unit, Faculty of Science and Technology, Universiti Kebangsaan Malaysia. A drop of bacterial broth was placed on a sterile coverslip for adherence and then incubated for 48 h. Following this, the coverslip with the biofilm sample was subjected to post-fixation in a 2.5% glutaraldehyde solution in PBS for approximately 1-2 h. The coverslip was then rinsed by washing twice with 1 mL of PBS and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min. Subsequently, the biofilms on the coverslips were dehydrated sequentially for 5 min in 50%, 70%, 90% and 100% ethanol. The biofilm sample were then subjected to critical point drying to transition the fluid to gas while preserving the biofilm structure. The dehydrated biofilm sample was mounted on an SEM stub and examined using a Variable Pressure Scanning Electron Microscope (VPSEM) model Zeiss/LEO1450VP at 15 k to observe any structural change.

TABLE 2. Primers sequence for genes studied

Gene	Associated virulence trait	Primers sequence	Tm (°C)	Reference
pilT	Motility	F:5′-CCGCGGATCCGGTAAGCCTCGGTGAAAGCC-3′ R:5′-CCGCGGATCCCCGTTAATCTTCATAGAGGG-3′	58	Clemmer, Bonomo & Rather (2011)
cpaA	Protease	F: 5′-CTGCTTTAGGAAAATGGG-3′ R: 5′-CGCCTTCAATCATTCTAAG-3′	55	Tilley et al. (2014)
katG	Catalase	F: 5′-GGCGATGAAAAAGAATGGTTA-3′ R: 5′-ATTTCTTCATCATCCATTGCC-3′	56	Sato et al. (2019)
bap	biofilm	F: 5′-ATGCCTGAGATACAAATTAT-3′ R: 5′-GTCAATCGTAAAGGTAACG-3′	55	Modarresi et al. (2015)

RESULTS AND DISCUSSIONS

ANTIBIOGRAM

Among the 14 hospital isolates examined, a notable trend has emerged where 100% exhibit resistance to ampicillin, erythromycin, cefazolin, ciprofloxacin and chloramphenicol. Additionally, 57% show resistance to tetracycline, 50% to gentamicin, 79% to imipenem and 43% to polymyxin B. It is particularly alarming that both strains Ab_H4 and Ab_H10 exhibit resistance to all tested antibiotics. This study aligns with findings reported by Lima et al. (2020), which highlighted a troubling increase in resistance, especially to polymyxin drugs. These results suggest a high level of genetic adaptation and robust resistance phenotypes in these strains, warranting increased attention.

The MIC of aspirin was determined to be 6.25 mg/mL. Upon treatment with ½ MIC aspirin, the antibiotic susceptibility profile of the Ab_H4 and Ab_H10 isolates were reassessed following an overnight incubation with a sub-minimum inhibitory concentration (sub-MIC) of 3.25 mg/mL aspirin. Treatment outcomes showed a restoration of susceptibility to cefazolin, imipenem and polymyxin B, alongside persistent resistance to ampicillin and erythromycin. Zimmermann and Curtis (2018) discussed the varying impact of aspirin on antibiotic susceptibility among different organisms, noting both increased resistance and susceptibility in its presence. Herewith, this study demonstrates that aspirin administration can enhance certain antibiotic susceptibility, shedding light on its potential to address antibiotic-resistant strains of *A. baumannii*.

The regained susceptibility to cefazolin and imipenem, key antibiotics in treating Gram-negative bacterial infections, indicates that exposure to sub-MIC aspirin can re-establish the efficacy of these antibiotics against both isolates. The recovery of susceptibility implies that aspirin may counter specific resistance mechanisms employed by these isolates, possibly through interactions with the bacterial cell membrane or modulation of efflux pumps (Alfizah, Sarah & Noraziah 2015; Kyriakidis et al. 2021). These findings suggest pleiotropic effects of aspirin extending beyond its well-known anti-inflammatory actions.

However, the sustained resistance to ampicillin and erythromycin, even after sub-MIC aspirin exposure, underscores the intricate nature of antibiotic resistance mechanisms in *A. baumannii*. Aspirin's response to bacteria may be specific to certain antibiotic classes, and the continued resistance to both ampicillin and erythromycin could be associated with mechanisms unaffected by aspirin exposure. This underscores the diverse range of antibiotic resistance mechanisms, emphasising the need for a comprehensive strategy to combat antibiotic-resistant pathogens.

A significant outcome is the restoration of susceptibility to polymyxin B, often a last-resort treatment for multidrug-resistant *A. baumannii* infections. The observed recovery implies that aspirin may have a synergistic effect with polymyxin B, potentially enhancing its antibacterial activity. Recent studies by Li et al. (2022) support this, emphasising the effectiveness of treating *A. baumannii* infections through the synergistic combination of polymyxin B and the drug ceftazidime. This intriguing prospect demands further investigation to assess the potential synergy between aspirin and polymyxin B, potentially leading to the development of more effective combination therapies.

MOTILITY ASSAY

Among the analysed isolates, 5 strains, namely Ab_H2, Ab_H3, Ab_H5, Ab_H7 and Ab_H9, obtained collectively from blood samples, exhibited high motility characteristics. This aligns with the findings of Vijayakumar et al. (2016), who observed a significant increase in motility traits among strains isolated from blood. Despite displaying lower motility in comparison to blood isolates, the two strains identified as highly virulent, Ab_H4 and Ab_H10, were subjected to analysis for motility effects following sub-MIC aspirin treatment. The results showed a reduction in motility; however, the decrease was not deemed significant,

indicating a complex interaction between aspirin and *A. baumannii* motility-related factors.

PROTEASE ASSAY

Based on the test results, four isolates, namely Ab_H3, Ab_H5, Ab_H8 and Ab_H10, exhibited the ability to secrete extracellular protease, which could play a role in the virulence of these bacterial strains. These strains were obtained from two sources, namely tracheal aspirate and blood. Interestingly, while some isolates demonstrated the ability to secrete protease, there were also isolates unable to do so, even when isolated from the same anatomical location. It can be concluded that the secretion of protease is not limited to specific infection sites within the body. Following overnight incubation with a sub-MIC of aspirin, the level of protease secreted by protease-producing strains has reduced significantly. This finding shows the ability of aspirin to modulate protease secretion in the isolates.

Proteases are known to play a crucial role in *A. baumannii* pathogenesis by facilitating tissue invasion, immune evasion and the breakdown of host defenses (Antunes et al. 2011). The observed decrease in protease secretion in response to sub-MIC aspirin exposure highlights a potential mechanism by which aspirin might interfere with the virulence machinery of *A. baumannii*. This finding suggests that aspirin could exert an inhibitory effect on key regulatory pathways or factors involved in protease production, leading to a diminished virulence phenotype in these isolates.

CATALASE ASSAY

An investigation involving the treatment of selected *A. baumannii* isolates: Ab_UKMCC (environmental control isolate), Ab_H4 and Ab_H10 with sub-minimum inhibitory concentration (sub-MIC) aspirin at a concentration of 3.25 mg/mL showed a significant reduction in catalase production. Catalase is a crucial enzyme utilised by *A. baumannii* species to neutralise reactive oxygen species produced by host defences, thereby enhancing the bacteria's resistance to oxidative stress encountered during infections (Sun et al. 2016). The observed reduction in catalase production following sub-minimum inhibitory concentration (sub-MIC) aspirin treatment highlights the potential of aspirin to interfere with the bacteria's ability to withstand oxidative stress. This indicates that aspirin may have the potential to modulate the main pathways or mechanisms responsible for catalase expression, consequently reducing *A. baumannii*'s ability to evade host immune responses (Wright et al. 2017). Interestingly, the environmental isolate, Ab_UKMCC, also showed a reduction in catalase production following sub-MIC aspirin exposure. This suggests that aspirin's effect on catalase regulation may not be limited to clinical isolates alone but may also influence isolates from the environment.

BIOFILM ASSAY

The identification of Ab_H1, Ab_H4, Ab_H8, Ab_H9, Ab_H10 and Ab_H11 as strong biofilm producers highlights the importance of biofilm formation in specific host environments, primarily tracheal aspirations where *A. baumannii* persists. The trachea's unique conditions, which consist of limited immune surveillance and stagnant fluid, make strong biofilm formation a protective strategy, allowing the bacteria to attach withstand clearance forces, and evade immune detection (Vijayakumar et al. 2016). However, among blood isolates, which are Ab_H2, Ab_H3, Ab_H5 and Ab_H7, weaker biofilm formation suggests a less preferred strategy for surviving in the bloodstream. This difference may be linked to the need for increased mobility in the bloodstream, minimising continuous cell mass formation to avoid immune attacks. The findings align with a study indicating low biofilm-forming ability in *A. baumannii* isolated from blood (Vijayakumar et al. 2016). Overall, the variations in biofilm formation reflect the bacteria's adaptive strategies to distinct host environments.

The significant reduction in biofilm formation observed in both Ab_H4 and Ab_H10 isolates following exposure to aspirin indicates the anti-biofilm effects of aspirin. For the Ab_H4 isolate, nearly complete biofilm eradication was achieved at the aspirin MIC, demonstrating the highly effective concentration of aspirin in disrupting biofilm integrity. The potential biofilm eradication by aspirin in the Ab_H4 isolate may be associated with aspirin's direct interference with biofilm matrix components or alterations in the regulatory pathways controlling its formation (El-Mowafy et al. 2014). The results of this study highlight the substantial potential of aspirin in combating biofilm-related infections caused by *A. baumannii*.

Sub-MIC aspirin showed a dose-dependent impact on reducing biofilm in Ab_H10, with a more significant effect at the MIC. This emphasises the concentration-dependent nature of aspirin's ability to inhibit biofilm in Ab_H10, underscoring the importance of optimising dosages for maximum therapeutic impact against *A. baumannii* infections. The different response of the environmental strain Ab_UKMCC to sub-MIC aspirin, promoting biofilm formation, raises questions about aspirin's role in *A. baumannii* strains with varying virulence, especially environmental isolates. However, almost complete eradication of biofilm in Ab_UKMCC achieved at higher aspirin concentrations (MIC aspirin) suggests the need to identify isolate origins before determining aspirin doses for biofilm treatment. Aspirin's potential to modulate biofilm formation in *A. baumannii* strains is a crucial finding, offering new possibilities for additional therapies targeting biofilm formation in challenging infections. Further investigations into the specific mechanisms of aspirin's anti-biofilm effects, including its impact on biofilm matrix and gene expression, are essential. *In vivo* studies are

needed to confirm aspirin's effectiveness and safety in real-life *A. baumannii* infections.

SEM ANALYSIS ON BIOFILM STRUCTURE

The scanning electron microscopy (SEM) analysis showed notable structural alterations in biofilms treated with aspirin. By referring to Figure 1, the isolate Ab_H4, subjected to sub-minimum inhibitory concentration (sub-MIC) aspirin treatment at 3.125 mg/mL, exhibited a significant disruption in biofilm structure. This was evidenced by the compromised overall integrity of the biofilm, marked by collapsed structures and the presence of only residual small structures.

Similar trends were observed for the isolate Ab_H10, albeit to a slightly lesser extent. As shown in Figure 2, at the sub-MIC of aspirin, 3.125 mg/mL, a discernible disruption in the biofilm structure of Ab_H10 was noted, albeit to a lesser degree compared to Ab_H4. This emphasises the possibility of varying susceptibility among different isolates to aspirin treatment, highlighting the requirement for a nuanced strategy when contemplating the utilisation of aspirin for disrupting biofilms.

These findings are particularly significant in the context of addressing the challenges posed by *A. baumannii* infections, which are notorious for their antibiotic resistance and biofilm-associated virulence. The observed disruption in biofilm structure upon aspirin treatment holds promise as a potential strategy to weaken the pathogenicity of *A. baumannii.* By targeting the biofilm structure, aspirin may not only impact the physical architecture but also potentially attenuate the bacterium's ability to establish persistent infections (El-Mowafy et al. 2014).

PRESENCE OF VIRULENCE-ASSOCIATED GENE

The presence of virulent-associated genes in two most virulent strains, Ab_H4 and Ab_H10, was then analysed. The *bap* and *katG* genes were present in these two isolates, which were responsible for biofilm formation and secretion of catalase, respectively (Figure 3). However, no motility-associated gene, *pilT*, was detected in the isolates despite being motile. The absence of the *pilT* gene is coherent with Harding, Hennon and Feldman (2018) in the *A. baumannii* M2 strain, which showed that pili Type IV encoded by the *pilT* gene is only needed for twitching motility and not surface-associated motility, which was assessed in this study. The complex genetic interactions required for surface-associated motility in *A. baumannii* were first explored by Blaschke, Skiebe and Wilharm (2021) and warrant further analysis in these strains in future research.

Despite both Ab_H4 and Ab_H10 isolates showing the ability to secrete protease, the *cpaA* gene, which is associated with protease production, was only found in Ab_H4 and not Ab_H10 (Figure 4). This indicates a possible different gene that is responsible for protease secretion in Ab_H10 and highlights the complex genomic interaction in the expression of virulence phenotypes of *A.baumannii* strain.

Analysing the presence of these virulence genes is important due to the diversity of *A. baumannii* strains observed in various clinical isolates. Across continents, different international clones have been identified and local isolates have undergone the development of clones to better adapt to their respective environments by acquiring or regulating genes (Biglari et al. 2017; Kabic et al. 2023). Therefore, it is crucial for periodic monitoring to be conducted on local isolates to ensure that the presence of this virulence gene is not rapidly transferred within intra-strain bacteria or between different bacterial species.

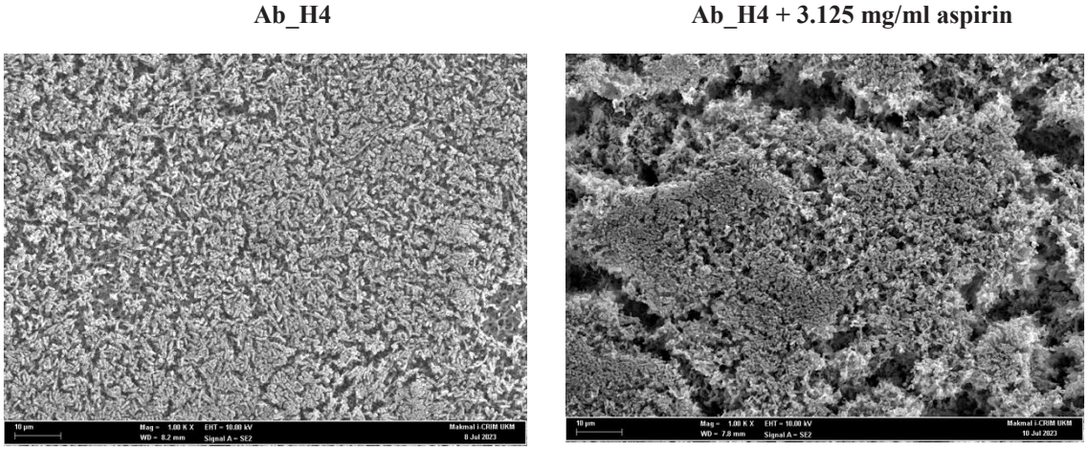

FIGURE 1. Biofilm structure of Ab_H4 upon treatment with aspirin

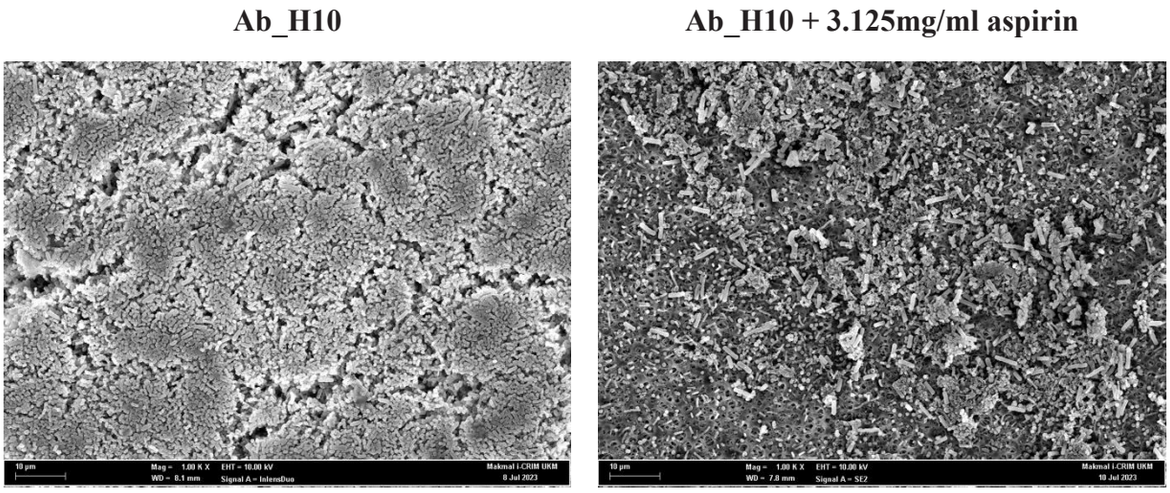

FIGURE 2. Biofilm structure of Ab_H10 upon treatment with aspirin

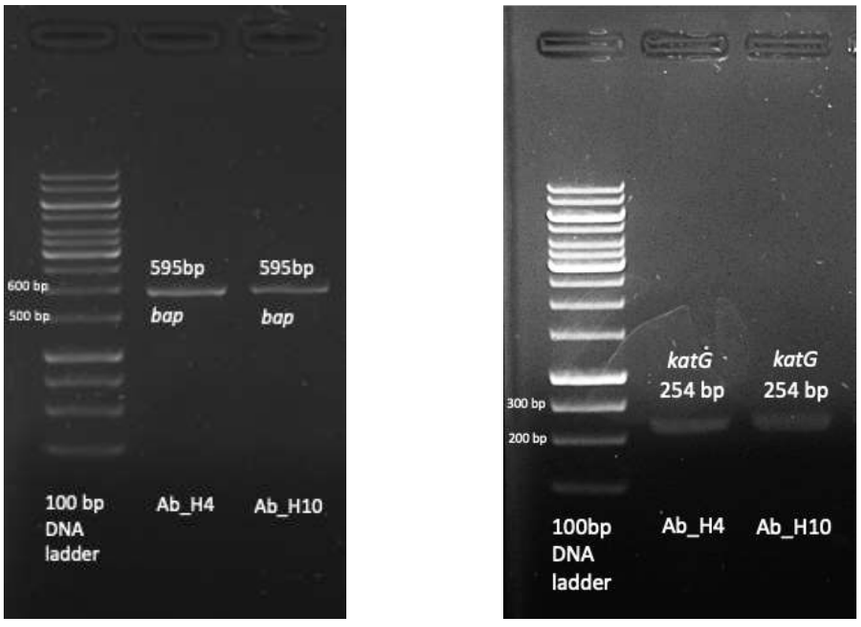

FIGURE 3. Gel electrophoresis shows the presence of virulence-related genes in both isolates Ab_H4 and Ab_H10. (a) Presence of *bap* gene (595 bp) and (b) Presence of *katG* gene (254 bp)

(a) (b)

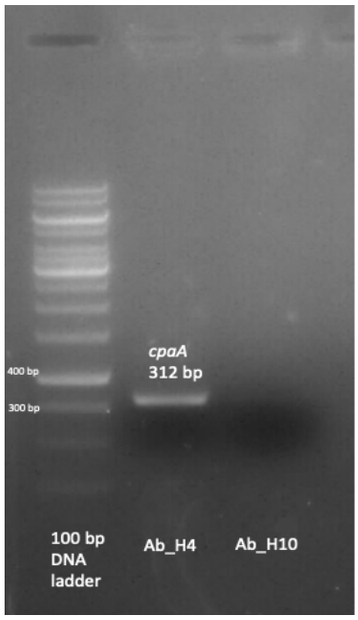

FIGURE 4. Electrophoresis gel showing the presence of *cpaA* gene in Ab_H4 but not in Ab_H10

CONCLUSION

In conclusion, this study has provided valuable insights into the antibiogram and virulence traits of *Acinetobacter baumannii* strains isolated from patients at Tuanku Muhriz UKM Chancellor's Hospital (HCTM). The alarming resistance to multiple antibiotics among these isolates underscores the urgent need for effective treatment strategies against this pathogen. In this study, two strains, Ab_H4 and Ab_H10, emerged as particularly virulent, displaying a complex interplay of various virulence factors, including resistance to all antibiotics, high motility, secreting protease, high catalase production, and strong biofilm formation. The correlation between the presence of specific genes and the observed virulence traits provides valuable molecular insights. The involvement of genes such as *pilT*, *bap*, *katG*, and *cpaA* in different strains highlights the intricate genetic mechanisms underlying *A. baumannii's* pathogenicity. The innovative exploration of aspirin as an antivirulence agent demonstrated promising results. The restoration of susceptibility to previously resistant antibiotics, disruption of biofilm formation, and reduction in enzyme secretion upon aspirin treatment opens new avenues for tackling *A. baumannii* infections. While no significant effect on bacterial motility was observed, the potential of aspirin as an adjunct therapy alongside antibiotics presents an exciting direction for further research. These findings underline the virulent capabilities of the hospital-isolated *A. baumannii* strain and the high potential of aspirin as an antivirulence agent.

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