



Genomic and phenotypic analysis of multidrug-resistant *Acinetobacter baumannii* focusing on efflux pump activity and biofilm-associated genes

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Received: 12 November 2025 / Accepted: 7 April 2026
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Abstract

Background *Acinetobacter baumannii* evolved as a critical pathogen of global concern due to its persistence and pathogenicity. To elucidate the underlying resistance mechanism, the role of efflux pumps and biofilm-associated genes was investigated. Furthermore, the genes that serve as potential targets or biomarkers were identified, offering valuable insights for the development of novel antimicrobial agents and optimization of antibiotic-based therapies.

Materials and methods Nineteen *A. baumannii* isolates were analysed for antibiotic resistance, efflux pump functionality, and biofilm formation. The ABS2 isolate underwent whole-genome sequencing (GenBank: JBJJNX000000000.1) to characterize resistance and virulence determinants, including the oxidative stress repair gene *yajL*, using the FunRich enrichment tool.

Results All isolates exhibited multidrug resistance, showing 100% resistance to ceftazidime and imipenem, with functional efflux pumps detected in every isolate using cartwheel method. Biofilm formation, assessed by the tissue culture plate method, was observed in 89% of isolates. PCR analysis revealed high prevalence of resistance genes *bla*_{OXA-51} (100%), *Int1-1* (79%) and *Int1-2* (15%). Efflux pump genes *adeA* (89%), *adeB* (100%), and *adeC* (63%) were present, while biofilm-associated genes *cusE*, *ompA*, *pgaA*, and *abaI* were also detected. Whole-genome and enrichment analyses of ABS2 revealed a diverse resistome and virulome contributing to oxidative stress resistance and immune evasion.

Conclusion The study highlights the high prevalence of biofilm formation and efflux pump-mediated resistance among *A. baumannii* isolates. Genomic insights into the ABS2 isolate emphasize its adaptability to hostile environments and potential to cause persistent infections, underscoring the global health threat posed by multidrug-resistant, biofilm-forming *A. baumannii*.

Keywords *Acinetobacter baumannii* · Multidrug resistance · *AdeABC* · Genome sequencing · Enrichment analysis

Introduction

Biofilm formation in bacteria is an important strategic approach exhibited under adverse conditions. They are formed by the aggregation of bacterial cells, resulting in the development of a multicellular mode of living, enabling them to gain a survival advantage in an unfavorable environment over their planktonic counterparts [1]. Biofilms are composed of various components, including extracellular polysaccharides (EPS), proteins, and extracellular DNA (eDNA), held together as a matrix within which microbial communities exist as microcolonies. Generally, biofilms are detrimental because biofilm-forming pathogens are reported to cause severe, chronic, and difficult-to-treat infections in

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hospital settings, particularly using contaminated health-care devices. They are also reported to be problematic in the food, dairy, and potable water supply industries [2]. The phenotypic alterations that bacteria experience in biofilms enable them to develop resistance to harmful environmental elements, such as antibiotics. Among the pathogenic bacteria, *Acinetobacter baumannii* is a typical opportunistic pathogen that contributes significantly to hospital-prone infections. *A. baumannii* tops the list of pathogenic bacteria that cause significant threats to human health and is included as a Priority 1-critical pathogen. It has also been included as an important pathogen in the ESKAPE cohort, which is mainly, but not necessarily, associated with nosocomial infections [1, 3].

The capability of *A. baumannii* to produce biofilms contributes to its persistence and viability in hospital settings. Moreover, these bacteria have developed resistance to high-end antibiotics, including carbapenems and colistin, limiting the choice of therapy and increasing morbidity and mortality. Antimicrobial resistance (AMR), together with its virulence profile, enhances *A. baumannii*'s natural competence in unfavorable environments [4]. *A. baumannii* employs many strategies, such as the production of hydrolyzing and modifying enzymes, efflux pumps, stress regulators and decreased permeability to combat and thrive antibiotics. Efflux pumps help bacteria route antibiotics outward, preventing them from having an impact on the cells. Minimum inhibitory concentrations (MICs) rise when antibiotics are released from cells, but drug accumulation decreases. This is because efflux pumps trigger the expulsion of antibiotics from cells, which lowers the drug entrapment and raises the MIC [5, 6]. Integrons are part of mobile genetic elements, and efflux pump genes, such as *Int1-1*, *Int1-2*, *adeA*, *adeB*, and *adeC*, are commonly linked to drug resistance in *A. baumannii*. Drug-resistant *A. baumannii* also widely inherits the *bla_{CTX}* and *bla_{OXA}* genes, which encode ESBL and carbapenem resistance. The predominant reported genes for biofilm production in *A. baumannii* include the outer membrane protein gene *ompA*, the pilus formation gene *cusE*, the polysaccharide gene *pgaA*, and the quorum sensing gene *AbaR* [7–9].

Whole genome sequencing of bacterial isolates provides detailed information about their species specificity, etiological factors, resistance, and virulence profiles. The bioinformatics analysis of bacterial genomes also helps identify new drug targets for drug discovery, aiding in the combat of drug resistance. Bacterial WGS also helps in comparing different genomes of the same species, which provides various insights about species evolution over time due to changes inherited by factors such as gene mutation, acquisition of resistance genes, and other metabolite genes responsible for its survival in various environments [10, 11]. In

clinical settings, the presence of biofilm makes it easier for the bacterial cells to resist antibiotic treatment. The present study aims to determine biofilm development and functional efflux pumps, and to relate their association with drug resistance, immune evasion and environmental stress tolerance using bacterial whole-genome sequencing of *Acinetobacter baumannii* clinical isolates.

Materials and methods

Antibiogram analysis of *A. baumannii* isolates

The antibiotic sensitivity test is a crucial method for determining the appropriate antibiotic selection and dose for therapeutic options in clinical settings, particularly for treating bacterial infectious diseases. Nineteen (19) *A. baumannii* isolates were obtained from the Voluntary Health Service Hospital, Chennai. The isolates were screened for antibiotic susceptibility, efflux pump activity, and biofilm formation. Disc diffusion test was carried out with the antibiotics Piperacillin (PI) (100 µg), Piperacillin-Tazobactam (PIT) (100/10 µg), Ceftazidime (CAZ) (30 µg), Levofloxacin (LE) (5 µg), Cefepime (CPM) (30 µg), Cefotaxime (CTX) (30 µg), Imipenem (IPM) (10 µg), Meropenem (MRP) (10 µg), Tetracycline (TET) (30 µg), Gentamicin (GEN) (10 µg), Amikacin (AK) (30 µg) and Ciprofloxacin (CIP) (5 µg) and the results were interpreted as per the CLSI guidelines [12].

Efflux pump detection by the cartwheel method

Efflux pumps are gatekeeper proteins in bacteria that facilitate the expulsion of harmful substances, including antibiotics, from cells. This function may inherit antibiotic resistance, rendering it a crucial focus in microbiological research. The cartwheel approach [13] to identifying efflux pumps was performed using uniformly dispersed solutions (10^6 cells per ml of broth) of nineteen *A. baumannii* isolates, which were streaked onto sterile Muller Hinton agar (MHA) plates containing ethidium bromide (EtBr-1 mg/liter) and incubated at 37 °C for 24 h. The cultured plates were examined with a UV transilluminator to detect fluorescence.

Efflux pump inhibition assay using carbonyl cyanide 3 chlorophenylhydrazone (CCCP)

The efflux pump inhibition assay was employed to validate the efflux activity of *A. baumannii* isolates [5]. The minimum inhibitory concentration (MIC) of antibiotics exhibiting increased resistance, specifically imipenem and meropenem for *A. baumannii*, was ascertained using the broth microdilution method in accordance with CLSI guidelines [12].

100 μl of both antibiotics, with concentrations ranging from 256 $\mu\text{g/ml}$ to 0.5 $\mu\text{g/ml}$, was introduced into each well of a microtiter plate containing 100 μl of sterile Muller Hinton broth (MHB) and 100 μl of *A. baumannii* culture suspension (10^6 cells per ml of broth) added to each well along with 10 μl of resazurin dye. The plates were incubated at 37 °C for 24 h. The color change from blue to pink indicates bacterial growth. The positive control wells contained MHB medium and bacterial suspension, while the negative control wells comprised culture medium and antibiotics. The reference strain *Escherichia coli* ATCC 8739 served as the positive control. The similar procedure was conducted with 0.25 $\mu\text{g/mL}$ CCCP present. A reduction of fourfold or more in the MIC values of antibiotics following the introduction of CCCP signifies efflux activity [14].

Biofilm detection by tissue culture plate method

Biofilms can form on a variety of surfaces that offer favorable conditions for survival, including medical implants, living tissues, and other natural habitats, making them a significant factor in both hospital-aided infections and environmental microbiology. The ability of *A. baumannii* isolates to develop biofilm was assessed using the polystyrene tissue culture plate method (TCP) [15]. A loopful of *A. baumannii* culture was inoculated into sterile Trypticase soy broth (TSB) supplemented with 1% glucose (TSB + G) (Himedia) and then incubated at 37 °C for 24 h. Following incubation, the *A. baumannii* culture medium was diluted with sterile TSB + G medium in a 1:100 ratio. About 150 μL of diluted culture was dispensed into each well of TCP. Following incubation for 24 h, each well was thoroughly rinsed three times with phosphate-buffered saline (PBS, pH 7.2). The biofilm biomass was fixed at 60 °C for 20 min and stained with 175 μl of 0.1% aqueous crystal violet stain (CV) for 5 min. Then, the TCP was washed with demineralized water to remove excess stain, and the optical density at 570 nm ($\text{OD}_{570\text{ nm}}$) of the adherent biofilm biomass was measured using a 96-well microtiter plate reader (Bio-Rad iMark). The average optical density (OD) of negative controls was computed, and three times standard deviation (SD) was added to establish the optical density cut-off (ODc). *A. baumannii* isolates exhibiting an optical density (OD) below the critical threshold (ODc) demonstrate inadequate biofilm formation capabilities. Isolates having OD values over 0.240 were designated as strong, ranging from 0.120 to 0.240 as moderate, and below 0.120 as weak biofilm producers, respectively.

Light microscopy of *A. baumannii* biofilm

Biofilms are protective matrices formed by bacteria, consisting of organic materials like proteins, polysaccharides, and nucleic acids. This makes biofilms up to a thousand times more resistant to drugs compared to free-floating planktonic bacteria. The different stages of biofilm formation in *A. baumannii* were analyzed utilizing a light microscope [16]. A 24-hour-old culture was resuspended in a freshly prepared TSB + G broth at a ratio of 1 in 100, and the resulting culture suspension was added into each well up to the brim of a sterile 12 well microtiter plate. Sterilized cover slips (2 cm \times 2 cm) were positioned atop the wells, followed by incubation at 37 °C. The coverslips were removed from each well at various time intervals and gently washed three times with neutral PBS solution. They were then subjected to staining for 10 min using aqueous CV (0.5%) stain. The residual stain on the coverslips was removed using desalinated water, and then the coverslips were air-dried. The morphological characteristics of developing biofilm were examined by observing the coverslips under a light microscope.

Stress response assay using hydrogen peroxide (H_2O_2) disc diffusion assay

The hydrogen peroxide assay was employed to evaluate oxidative stress damage induced by environmental stimuli in bacteria. The extensively drug-resistant and highly biofilm-producing *A. baumannii* ABS2 isolate and control strain *Escherichia coli* ATCC 8739 was evaluated for stress response using the H_2O_2 disc diffusion experiment [17]. A mid-log phase culture was swabbed onto sterile Luria Bertoni agar plates, and sterile 6 mm filter paper discs incorporated with 10 μl of freshly prepared H_2O_2 at 2 M concentration were placed. The plates were subsequently incubated at 37 °C for 24 h. The formation of distinct zones around the discs indicates the susceptibility of the bacterial strains to H_2O_2 .

Detection of resistance and virulence-associated genes by PCR

The polymerase chain reaction is a well-established method that facilitates the amplification of specific genes of interest. It serves as a crucial function in diagnostics, forensics, and scientific research. Genomic DNA from each *A. baumannii* isolate was extracted using the phenol-chloroform method, following the protocol of Sambrook et al. [18]. Specific primers targeting selected genes (listed in Table 1) were synthesized by Barcode Biosciences (Bangalore, India). PCR amplification was carried out in a 10 μL reaction volume, which consisted of 5 μL of 2x red dye Taq

Table 1 Sequence of Specific primers and PCR conditions used to amplify virulence and resistance genes

Target genes	5' – 3' and 3'-5' sequence of primers	Amplification size (bp)	Initial denaturation condition	Denaturation conditions for 35 cycles	Annealing conditions for 35 cycles	Extension condition for 35 cycles	Final extension condition	References
<i>abaI</i>	F-AATGCCTATTCCCTGCTCAC R-ATTGCTTCTTGAGAATTGC	132	94°C for 3 min	94°C for 0.30 min	55°C for 0.30 min	72°C for 0.45 min	72°C for 7 min	[8]
<i>pgaA</i>	F-GCCGACGGTCGCGATAC R-ATGCACATCACAAAACGGTACT	150	94°C for 3 min	94°C for 0.30 min	58°C for 0.30 min	72°C for 0.45 min	72°C for 7 min	
<i>ompA</i>	F-CGCTTCTGCTGGTGTGAAT R-CGTGCAGTAGCGTTAGGGTA	531	94°C for 3 min	94°C for 0.30 min	58°C for 0.30 min	72°C for 0.45 min	72°C for 7 min	
<i>cusE</i>	F-TCAGACCCGGAGAAAACTTAA CG R-GCCGGAAGCCGTAT GTAGAA	150	94°C for 3 min	94°C for 0.30 min	58°C for 0.30 min	72°C for 0.45 min	72°C for 7 min	
<i>bla_{oxA-51}</i>	F-TAATGCTTTGATCGGCCTTG R-TGGATTGCACTTCATCTTGG	353	94°C for 3 min	94°C for 0.30 min	55°C for 0.30 min	72°C for 0.45 min	72°C for 7 min	
<i>Int1-1</i>	F- CAGTGGACATAAGCCTGTTC R- CCCGAGGCATAGACTGTA	160	94°C for 3 min	94°C for 0.30 min	58°C for 0.30 min	72°C for 0.45 min	72°C for 7 min	[9]
<i>Int1-2</i>	F- GTAGCAAACGAGTGACGAAATG R- CACGGATATGCGACAAAAGGT	788	94°C for 3 min	94°C for 0.30 min	62°C for 0.30 min	72°C for 0.45 min	72°C for 7 min	
<i>ade C</i>	F- TACGGACTGCTACGCTTAAT R- AACAGGATGACCTGCTAACA	527	94°C for 3 min	94 °C for 0.30 min	55°C for 0.30 min	72°C for 0.45 min	72°C for 7 min	[7]
<i>ade B</i>	F- TTAACGATAGCGTTGTAACC R- TGAGCAGACAATGGAATAGT	541	94°C for 3 min	94 °C for 0.30 min	53°C for 0.30 min	72°C for 0.45 min	72°C for 7 min	
<i>ade A</i>	F- ATCTTCCTGCACGTGTACAT R- GGCGTTCATACTCACTAACC	513	94°C for 3 min	94°C for 0.30 min	55°C for 0.30 min	72°C for 0.45 min	72°C for 7 min	
<i>rpoB</i>	F-TAYCGYAAAAGAYTTGAAAGAAG R- CMACACCYTTGTTMCCRTGA	350	94°C for 3 min	94°C for 0.30 min	55°C for 0.30 min	72°C for 0.45 min	72°C for 7 min	[19]

DNA polymerase master mix (Bio-Rad, India), 1 µL of the DNA template, 3 µL of sterile molecular-grade water, and 0.5 µL of specific primers at a concentration of 10pmol. The primers for specific genes were annealed under the conditions provided in Table 1. Negative control includes a reaction mixture without DNA. The target genes were amplified using a Nexus Gradient Master cycler (Eppendorf, Germany). PCR products were separated by 1.5% agarose gels stained with ethidium bromide. Electrophoresis was carried out in 1x Tris-EDTA buffer at 50 V for 90 min. The amplified DNA fragments were visualized and documented using the Alpha Imager Mini gel documentation system (Protein Simple).

Genome library construction and bacterial whole genome sequencing (WGS)

Whole genome sequencing of bacteria provides signature information regarding their Metabolism and functionality. The incorporation of whole-genome sequencing studies can enhance our understanding of bacterial behaviour and resistance mechanisms. By studying the genes involved in biofilm formation, one can gain insights into bacterial survival strategies in various environments. The *A. baumannii* ABS2 isolate was chosen for whole genome sequencing investigation due to its resistance and biofilm formation characteristics. *A. baumannii* ABS2 DNA was isolated using the

DNeasy DNA extraction kit (QIAGEN, USA) following the manufacturer's procedure. DNA concentration was measured using a Nanodrop spectrophotometer (ThermoFisher, USA). Sequence libraries were constructed from extracted DNA and sequenced at high depths using the Illumina Nova-seq 6000 platform. Whole-genome sequencing was also carried out on the same platform. The acquired raw data were analyzed by FastQC [20] and MultiQC [21].

Raw reads underwent processing to eliminate adapter sequences and low-quality bases, utilizing default parameters with fastp v0.12.4 [22]. The reads that passed quality control were assembled into contigs utilizing Unicycler v0.5.0 [23]. Contigs with a length of less than 200 bp were excluded from the assembly process. The genome statistics were evaluated using QUAST v5.0.2 [24]. The genome assembly quality was assessed by aligning the reads into assembled contigs using the Bowtie platform version 2.4.5 [25]. The *A. baumannii* ABS2 genome completeness was assessed utilizing BUSCO version v5.3.2 [26] alongside bacteria odb10. The draft genome of the *A. baumannii* ABS2 isolate was deposited in the National Centre for Biotechnology Information (NCBI) database with the accession number BJJNX000000000.1.

Genome analysis and annotation

Taxonomic identification was conducted by Ribosomal Multilocus Sequence Typing (rMLST) [27]. The compiled contigs were annotated by a BLAST v2.13.0 [28] homology search against the NCBI database and utilizing Pokka v1.13 [29]. The Virulence Factor Database (VFDB) is used to identify the genes responsible for virulence, while the Comprehensive Antibiotic Resistance Database (CARD) is utilized to assess antibiotic resistance genes [30, 31]. Further, the processed genes were analyzed using a functional enrichment and network analysis tool FunRich [32].

Data analysis

Biofilm development in relation to antibiotic resistance among the isolates was analyzed using a chi-square test [9] using SPSS statistical software.

Results

Antibiotic resistance profile and efflux pump detection assay

The comprehensive examination of efflux pump activity alongside antibiotic sensitivity presents numerous benefits. The result enhances our understanding of microbial resistance mechanisms, which is crucial for developing effective treatments for antibiotic-resistant infections. This study revealed that *A. baumannii* isolates exhibited resistance to various classes of antibiotics, including penicillin, beta-lactams, carbapenems, and quinolones. The maximum resistance (100%) was detected towards ceftazidime and imipenem. About 89% of isolates were resistant to Piperacillin, Cefepime, Gentamicin, Piperacillin/Tazobactam, Amikacin, Cefotaxime, Tetracycline, and Levofloxacin. Meropenem and Ciprofloxacin resistance were observed among 84% and 78% of isolates, respectively (Fig. 1). All isolates of *A. baumannii* exhibited positive efflux pump activity, as evidenced by the absence of fluorescence under UV light at a 1 mg/L concentration of EtBr (Fig. 2a, b). The efflux pump inhibition assay performed with highly resistant antibiotics (imipenem and ceftazidime, supplemented with CCCP) showed a moderate decrease in the minimum inhibitory concentration (MIC) of the antibiotics (Fig. 2c, d, e, f). The *bla_{oxa}* gene's inheritance in all strains may hydrolyse beta-lactam antibiotics, while biofilms reduce membrane permeability. The efflux pump serves as one mechanism of antibiotic resistance in this study, though it is not the sole one.

Biofilm detection by tissue culture plate method

Investigating biofilms in multidrug-resistant pathogens is crucial for understanding chronic infections, as bacteria associated with biofilms tend to be more challenging to eliminate and can lead to persistent infections. Upon testing biofilm formation, 5% of the isolates were classified as strong biofilm producers, 84% as moderate biofilm producers, and 10% as non-biofilm producers (Fig. 3).

Light microscopy of *A. baumannii* biofilm

Light microscopic examination of *A. baumannii* revealed distinct biofilm development phases, including adhesion, colony formation, biofilm maturation, and dispersion. A unique morphology of the biofilm was observed at each stage over various periods. Biofilm progresses rapidly through each stage, transitioning from the free-floating stage to the mature biofilm stage. Bacteria present within biofilms were identified at the maturation phase. Organism multiplication and dissemination within the biofilm were observed during the colonization phase, leading to biofilm development (Fig. 4).

Stress response assay using hydrogen peroxide (H₂O₂) disc diffusion assay

The stress response assay employed to evaluate oxidative stress in cells resulting from detrimental effects, including antibiotic exposure and cellular damage. In this investigation, the control strain *Escherichia coli* ATCC 8739 exhibited a zone of inhibition measuring 20 mm in diameter, resulting from the action of catalase and peroxidase enzymes surrounding the disc, indicating the absence of growth (Fig. 5a). The test isolate *A. baumannii* ABS2 exhibited no zone of inhibition surrounding the disc, indicating tolerance to oxidative stress, which suggests its ability to survive in adverse environments (Fig. 5b).

Detection of resistance and virulence-associated genes by PCR

PCR has transformed medical diagnosis and scientific investigation in the detection of pathogens, genetic diseases, gene cloning, and gene expression studies. In this study, many specific genes of interest were amplified by the PCR technique. The presence of the RNA polymerase subunit B (*rpoB*) gene, used for species validation of *A. baumannii*, and the *bla_{oxa-51}* gene, which encodes carbapenem resistance, was detected in all the isolates. The biofilm-associated genes *abaI* (quorum sensing), *pgaA* (adhesion polysaccharide), *ompA* (outer membrane protein), and *cusE*

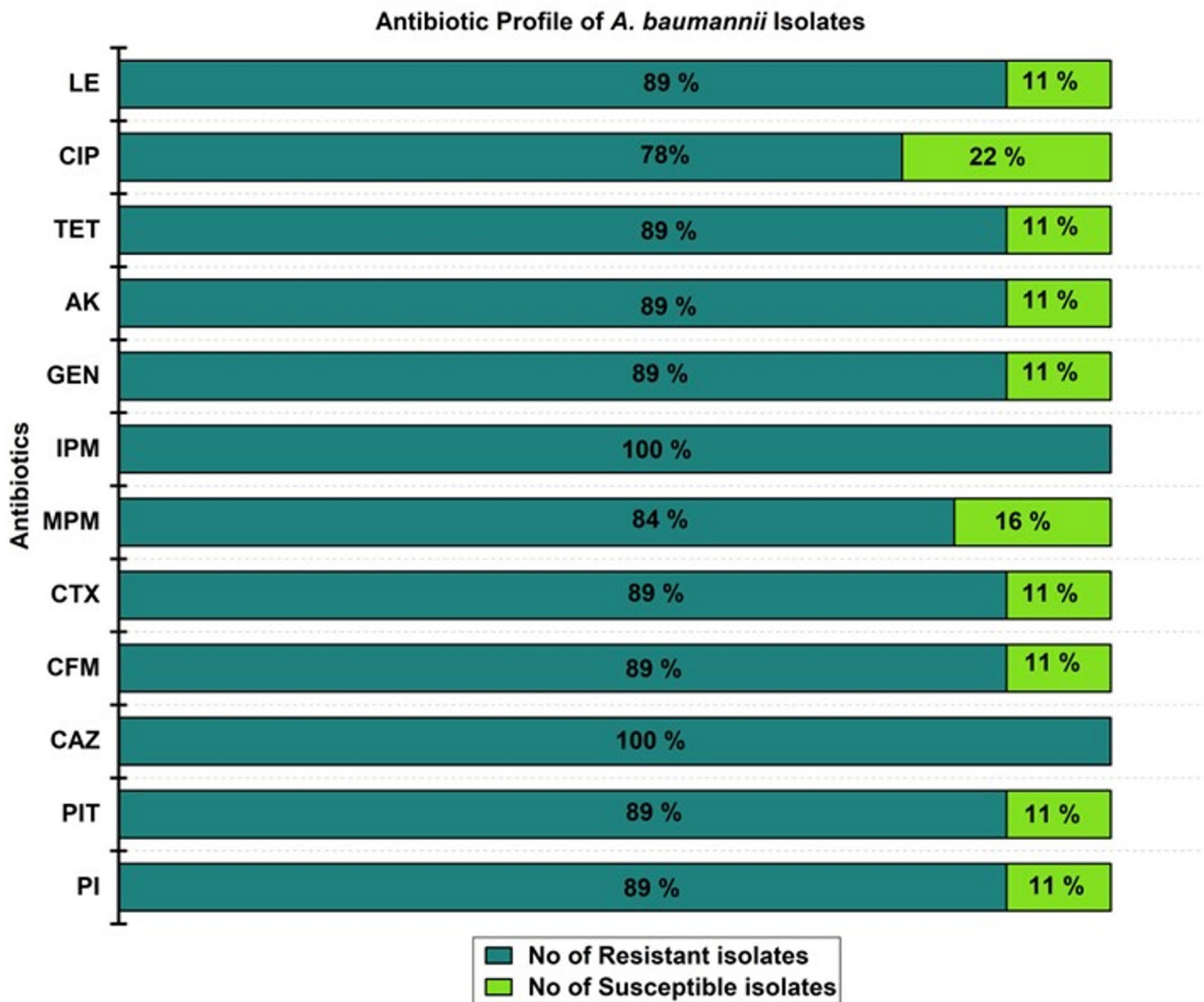


Fig. 1 The drug resistance pattern in *A. baumannii* isolates. Piperacillin-PI, Piperacillin/tazobactam-PIT, Ceftazidime-CAZ, Cefepime-CFM, Cefotaxime-CTX, Meropenem-MPM, Imipenem-IPM, Gentamicin-GEN, Amikacin-AK, Tetracycline-TET, Ciprofloxacin-CIP, Levofloxacin-LE

(pili attachment) were also observed in all (100%) of *A. baumannii* isolates. The efflux pump genes *adeA*, *adeB*, and *adeC* were present in 89%, 100%, and 63% of the isolates, respectively. The genes responsible for the transfer of drug-resistant genes, including class 1 and class 2 integrons, were present in 79% and 15% of isolates, respectively (Fig. 5).

Genome library construction and Bacterial whole genome sequencing (WGS)

In this study, whole-genome sequencing analysis helps in exploring the various genes responsible for antibiotic resistance and virulence in the *A. baumannii* ABS2 isolate. The obtained genome Length of *A. baumannii* ABS2 isolates is about 3,828,529 (3.8 MB), which is assembled into 60

contigs. The isolate *A. baumannii* ABS2 had a GC content of 38.95% and 3560 coding sequences, as determined using the Prokka pipeline (Supplementary Table S1, Fig. 6). Genome completeness, as assessed by BUSCO, was identified as 98.4%, and taxonomic identification, completed using rMLST, revealed that sample ABS2 belonged to *Acinetobacter baumannii* with 100% accuracy. The predicted genes included 1458 involved in Metabolism, 187 in Genetic Information Processing, 124 in Cellular Processes, 203 in Environmental Information Processing, and 74 in Organismal Systems. A total of 31 Antibiotic resistance genes were identified using the CARD database (Supplementary Table S2), and 59 genes were predicted as virulence factors according to VFDB, which included 14 biofilm formation genes, 1 outer membrane protein gene, and 2 Quorum

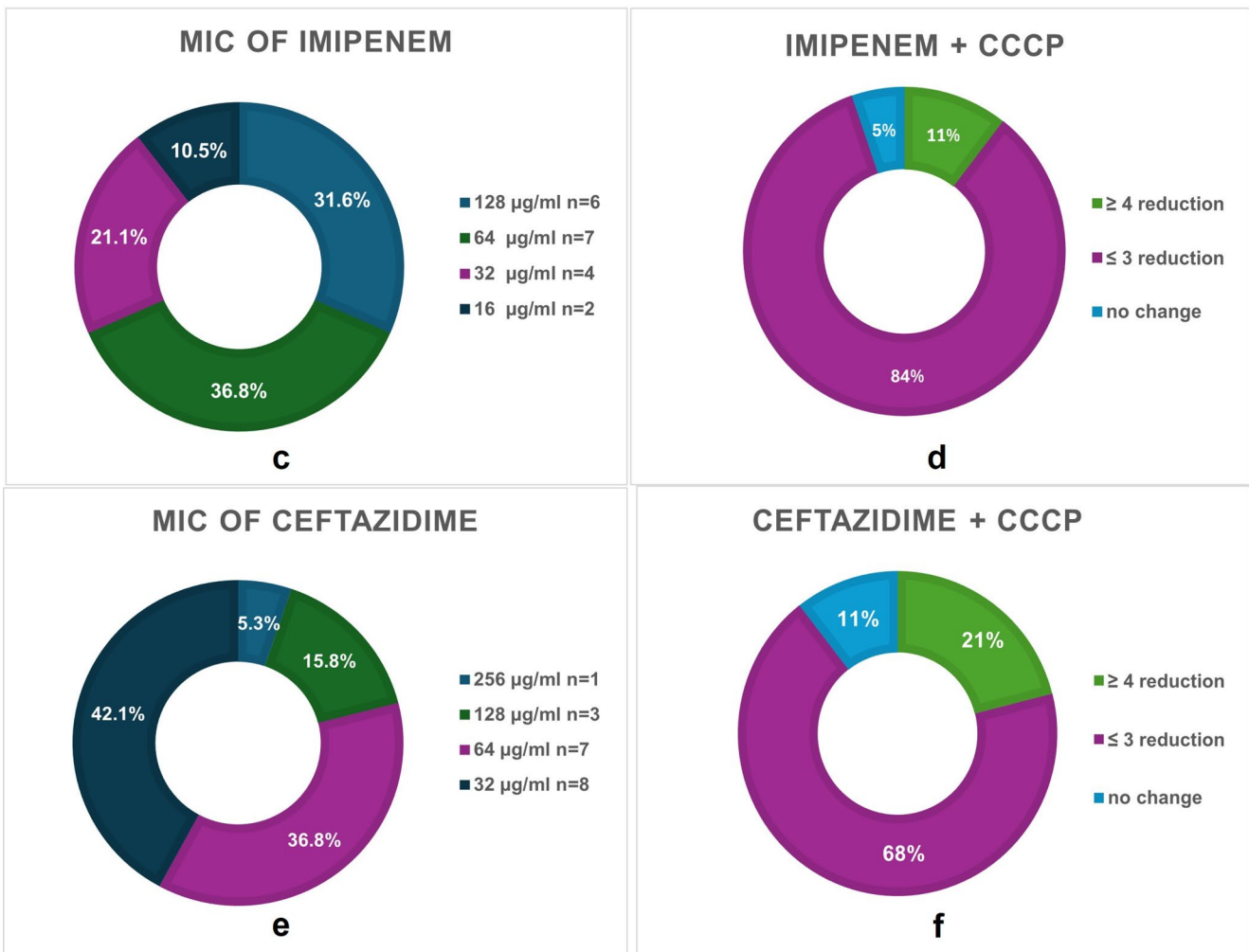


Fig. 2 Cartwheel method for efflux pump detection (a– Representative *A. baumannii* isolates in MHA plates with 1 mg/L of EtBr with efflux positive strains show no fluorescence because they pump the dye out, b—antibiotic-susceptible isolates of *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 29737, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 9621, *Shigella flexneri* ATCC

29508, *Salmonella typhimurium* ATCC 23564, *Staphylococcus aureus* ATCC 5388, *Proteus mirabilis* ATCC 25933 lacking efflux pumps with presence of fluorescence as a control). c MIC of imipenem at various concentrations and (d) MIC reduction of imipenem in presence of CCCP. e shows MIC of ceftazidime at various concentrations and (f) shows MIC reduction of ceftazidime in presence of CCCP

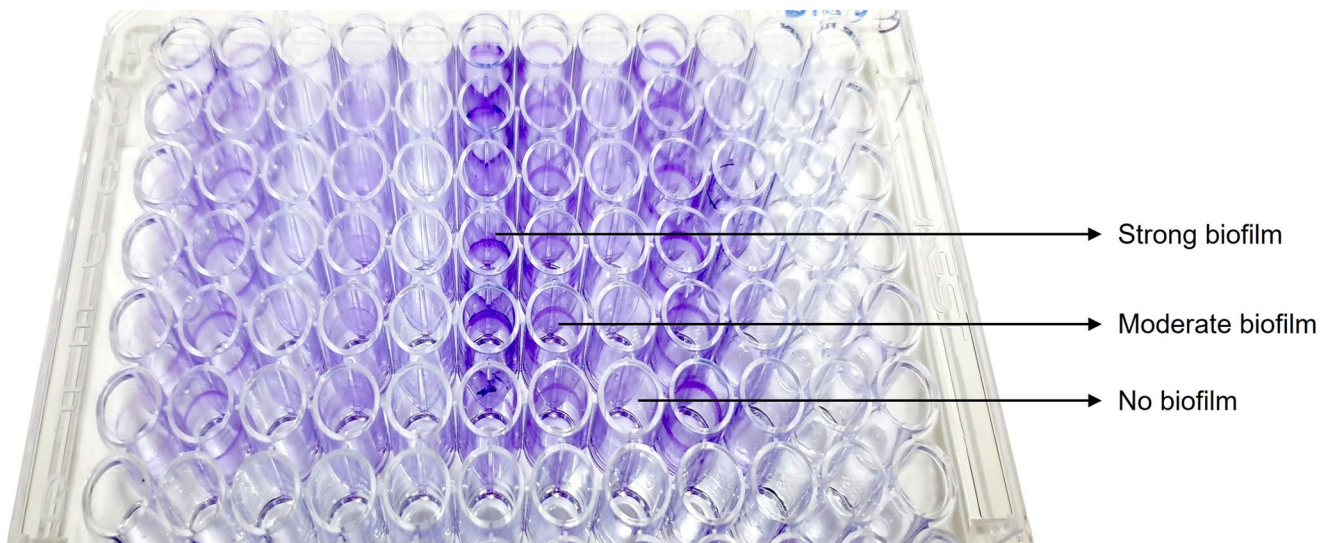


Fig. 3 Biofilm production using the tissue culture plate method, with *A. baumannii* phenotypes exhibiting no biofilm production, moderate, and strong biofilm adhesion in relation to biofilm development in TCP

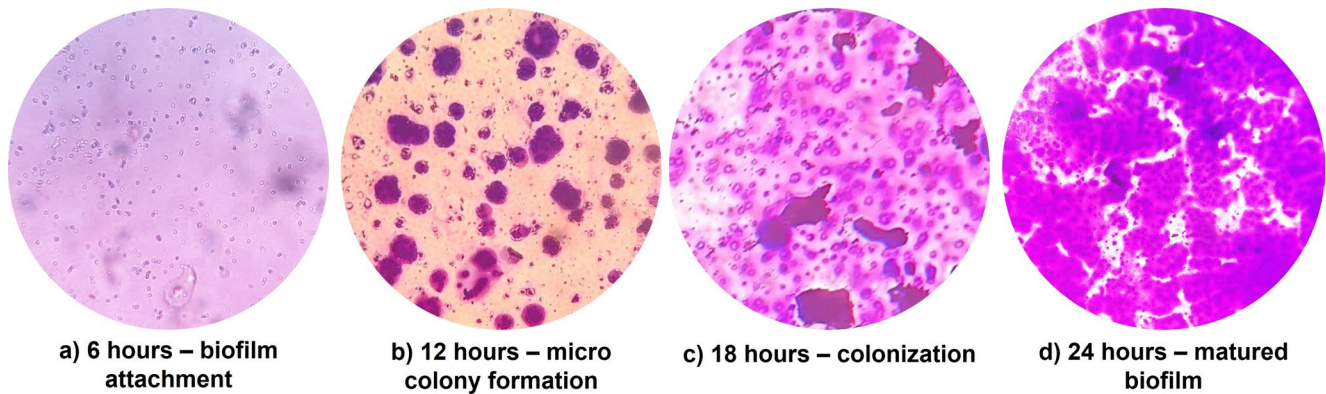


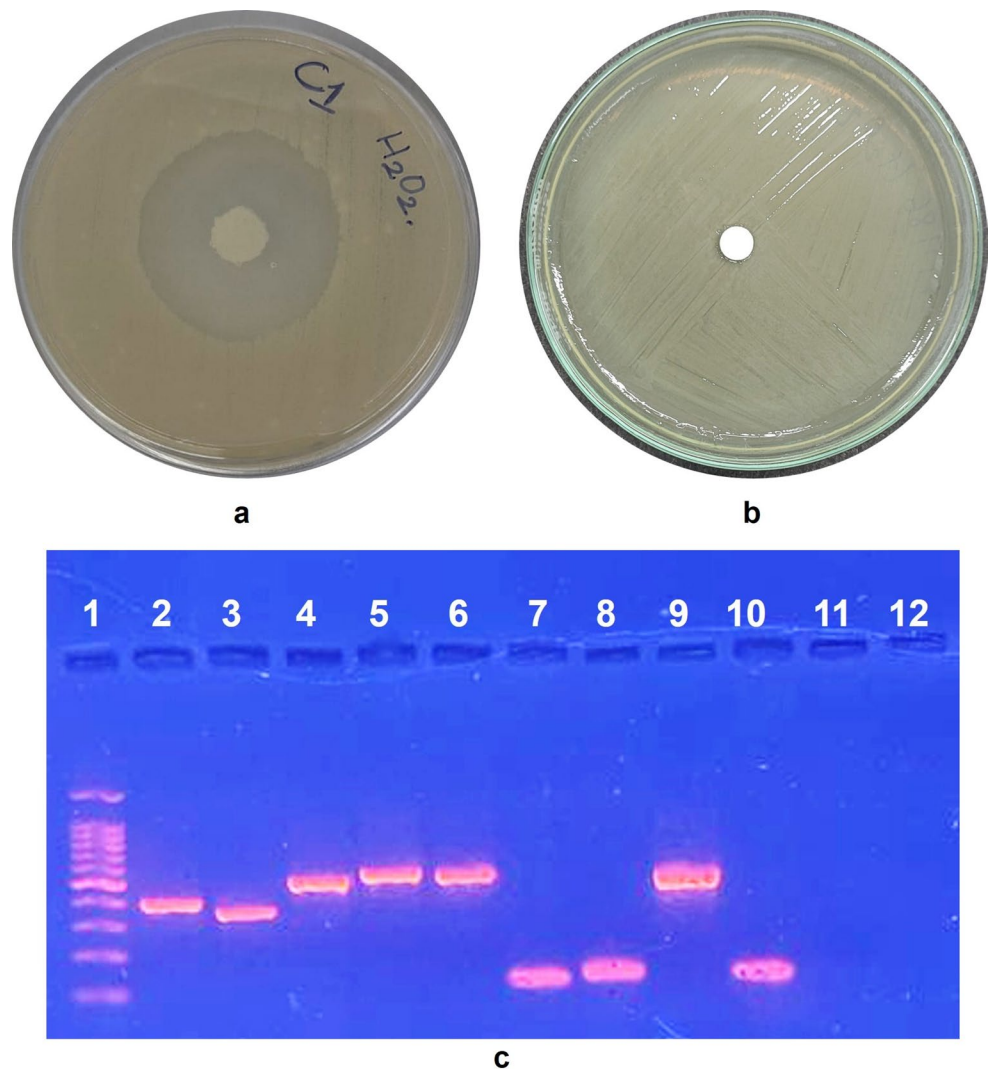
Fig. 4 Several stages of *A. baumannii* biofilm growth under a Light Microscope (total magnification 400X). The biofilm-coated CV-stained coverslip was analyzed at various time intervals: **a**—biofilm

attachment to the surface (6 h), **b**—microcolony formation along with water channel (12 h), **c**—Development of biofilm colony (18 h), **d**—matured biofilm (24 h)

sensing genes (Supplementary Table S3). The processed genes of ABS2 were analysed using the FunRich tool, which reveals protein domains associated with various functional cellular components of the genome. FunRich domain analysis of ABS2 (Fig. 7a) indicated an enrichment of protein domains associated with translation and DNA repair, specifically HhH1 (3.8%), tRNA-SAD (3.1%), S1 (3.1%), PCRF (2.3%), and ribosomal proteins S15 and L14 (2% each). The prevalence of ribosomal and tRNA synthetase domains indicates increased translational efficiency and protein turnover, whereas the enrichment of the HhH1 domain suggests enhanced DNA-binding and repair capabilities. The identified features enhance the isolate's survival in the presence of oxidative and antibiotic stress, aligning with the stress tolerance and resistance pathways revealed in the Reactome analysis. Venn diagram analysis of the *A. baumannii* ABS2 proteome (Fig. 7b), in comparison to the reference *A.*

baumannii (Accession number: GCF_025995075.1), identified a total of 625 shared proteins, which constitute the conserved core proteome. The ABS2 isolate contained 1,338 unique proteins, whereas 307 proteins were exclusive to the reference strain. The increased number of unique proteins in ABS2 indicates significant genomic variability, suggesting potential adaptation and functional diversification within the species. Pathway enrichment analysis conducted with FunRich indicated variations in functional representation between the *A. baumannii* reference proteome and ABS2. In Reactome pathway (Fig. 7c), the pathway Tolerance of reactive oxygen produced by macrophages exhibited the highest enrichment in both datasets, with values of 33.33% for the reference and 30.77% for ABS2, suggesting conserved mechanisms for oxidative stress tolerance. Moderate enrichment was noted for "Chorismate via Shikimate Pathway and Antimicrobial action and antimicrobial resistance

Fig. 5 (a) H₂O₂ disc diffusion assay for control strain showed zone of inhibition around the disc, (b) *A. baumannii* ABS2 isolate showed no zone around the disc due to tolerance to oxidative stress, c) PCR analysis of *A. baumannii* (ABS9). Lane: 1–100 bp marker DNA, 2-*rpoB* (350 bp), 3-*bla_{OXA-51}* (353 bp), 4-*adeA* (513 bp), 5-*adeB* (541 bp), 6-*adeC* (527 bp), 7-*abal* (132 bp), 8-*pgaA* (150 bp), 9-*ompA* (531 bp), 10-*cusE* (150 bp), [11-*Int 1* (160 bp), 12-*Int 2* (788 bp) were not amplified in ABS9 isolate]



in Mtb, with ABS2 exhibiting slightly higher percentages (19.23% and 15.38%) compared to the reference (16.67% and 12.50%). The pathway for cysteine synthesis from O-acetylserine exhibited negligible variation between the two conditions. ABS2 exhibited enrichment patterns that align with increased oxidative stress and potential for antimicrobial response. Protein–Protein interaction (PPI) network for the *yajL* gene (Fig. 7d), which is implicated in the cellular repair system, was generated from enrichment analysis. This network reveals interactions between *yajL* and various ribosomal proteins (e.g., *rpsA*, *rpsB*, *rplB*, *rplE*), chaperones (*dnaK*, *clpB*, *secA*), and metabolic enzymes (*sdhB*, *acnA*, *acnB*, *sucA*), suggesting its role in protein folding, oxidative stress response, and the maintenance of translation machinery.

Data analysis

The association between biofilm development and resistance patterns was evaluated using the Chi-square test and found to be statistically significant (p -value=0.015).

Discussion

Acinetobacter baumannii is a notorious opportunistic pathogen recognized as a leading cause of healthcare-associated infections. Being multidrug-resistant, *A. baumannii* remains a Critical Priority Pathogen according to the 2024 World Health Organization (WHO) list of bacterial priority pathogens [3]. This study reported that all *A. baumannii* isolates (100%) showed resistance to at least three antibiotic drugs. In contrast, other reports have ranged from 50 to 100%, with most of the resistance observed towards cephalosporins and carbapenems. These findings align with

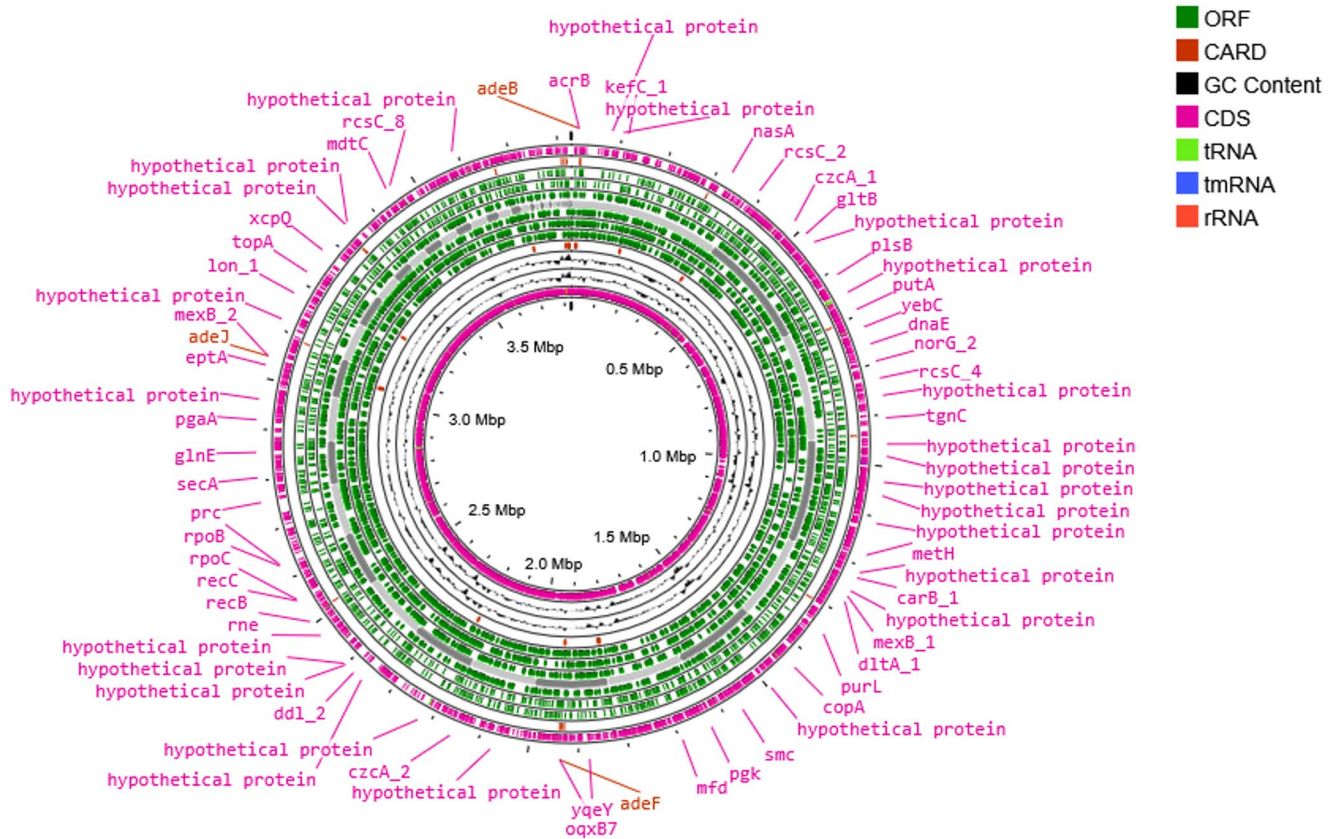


Fig. 6 Circular map of the assembled genome for the sample *A. baumannii* ABS2. The genome identified and predicted size is labelled with GC Content, CDS, and tRNA

previous experiments [33, 34]. The Ade ABC efflux pump of the RND class encodes drug resistance to beta-lactams, including carbapenems, chloramphenicol, fluoroquinolones, tetracycline, and aminoglycoside group of antibiotics. Approximately 100% of isolates were found to be positive for the phenotypically studied efflux pump in our study by Cartwheel method. The efflux pump genes *adeA* and *adeB* were more prevalent in our study compared to *adeC*, which was comparable to the previous study [9, 13]. This investigation demonstrated that *A. baumannii* isolates had greater resistance to the antibiotics hydrolysing imipenem and ceftazidime. Efflux pumps serve as a bridging mechanism in bacterial virulence and resistance. Efflux pumps are one of the mechanisms contributing to bacterial resistance, rather than being the sole factor. This study found that the incorporation of efflux pump inhibitors significantly enhanced antibiotic susceptibility; however, the effect was limited, likely due to the presence of additional resistance mechanisms, including the production of hydrolyzing enzymes, reduced membrane permeability due to biofilm formation, and modified target sites hosted by the bacteria. The results were concordant with previous reports [14].

In addition to the presence of efflux pumps, the ability of *A. baumannii* to form biofilms contributes to its increased

resistance to antibiotics [35]. In our study, approximately 89% of isolates demonstrated positive biofilm production using the microtiter plate method. Our study is consistent with other studies that indicate the tissue culture plate method to be an appropriate method for biofilm production tests [36]. The analysis using light microscopy of biofilm development unveiled the various stages involved in the process. The 6-hour phase was notably marked by surface adherence, whereas the 12-hour phase was associated with the synthesis of the polymer matrix and the development of small colonies. The mature and stabilized biofilms were noted at the 24-hour stage. The biofilm development phases in our study align with previous research findings observed among a few gram-negative bacteria [37]. Exposure of bacteria to antibiotics induces an oxidative stress response that regulates the production of numerous proteins and DNA repair mechanisms. In our work, the *A. baumannii* ABS2 isolate exhibited no sensitivity to hydrogen peroxide, likely attributable to its characteristics as a highly drug-resistant and strong biofilm producer. Previous research indicates that stress responses are significantly driven by the over-expression of efflux pumps and outer membrane proteins, which facilitate organism survival under stressful situations, such as persistent infections during antibiotic exposure [6].

for biofilm production, including *csuE*, *ompA*, and *pgaA*, in *A. baumannii* isolates are associated with biofilm development, attachment to the epithelial surface, and antibiotic resistance. Similar to our investigation, previous research studies also show a high prevalence of *cusE* (100%), *ompA* (81%), and *pgaA* (98%), respectively [9, 39]. The quorum-sensing gene *abaI*, responsible for the bacterial signaling mechanism, was found in all biofilm-forming *A. baumannii* isolates in our study. Previous studies have illustrated the widespread presence of approximately 83% of the *abaI* gene among *A. baumannii* clinical isolates and have also correlated its relationship with bacterial resistance [40].

In addition to PCR-based identification of resistance and virulence genes, the ABS2 isolate possessed high drug-resistant nature and high biofilm character it was subjected to whole genome sequencing (WGS). The WGS of *Acinetobacter baumannii* ABS2 isolate revealed numerous antibiotic resistance determinants, as identified through Comprehensive Antibiotic Resistance Database (CARD) analysis. The genome harboured four β -lactamase genes *ADC-73*, *OXA-23*, *OXA-66*, and *TEM-1* conferring resistance to β -lactam antibiotics, along with two fluoroquinolone resistance genes, *gyrA* and *parC*, and the aminoglycoside resistance gene *ANT(3'')-IIa*. WGS analysis also identified *msrE-mpheE*, which mediates macrolide and streptogramin resistance, and four additional aminoglycoside resistance genes *APH(3'')-Ib*, *APH(6)-Id*, *APH(3')-Ia*, and *armA*—localized within mobile genetic elements. Notably, the *armA* gene is known to confer high-level aminoglycoside resistance in *A. baumannii* [41, 42]. The *lpsB* gene, predicted in the ABS2 genome, contributes to intrinsic resistance to peptide antibiotics such as colistin through mutation or structural modification [43]. Multiple efflux pump systems that enhance antibiotic tolerance by actively extruding antimicrobial agents were also identified using WGS. These included genes from the RND (resistance–nodulation–cell division), MFS (major facilitator superfamily), and SMR (small multidrug resistance) families. The RND-type transporters *adeC* and *adeR* are associated with tetracycline resistance, while *adeL*, *adeF*, *adeG*, and *adeH* mediate resistance to fluoroquinolones and tetracyclines. Similarly, *adeI*, *adeJ*, *adeK*, and *adeN* confer resistance to tetracyclines, carbapenems, cephalosporins, macrolides, lincosamides, rifamycins, and phenicols, whereas *acrAB-tolC* is linked to tobramycin and colistin resistance. Within the MFS family, *AmvA* confers resistance to disinfectants, antiseptics, and macrolides, *AbaF* mediates phosphonic acid resistance, and *tetR* and *tet(B)* confer tetracycline resistance. The SMR-type efflux pump *abeS* is associated with resistance to macrolides and aminocoumarins, while the *macAB-tolC* operon, homologous to *acrAB-tolC*, belongs to the ABC transporter family and provides additional macrolide resistance [35]. The

coexistence of these multiple resistance determinants in the ABS2 genome indicates that *A. baumannii* possesses the genetic potential to evolve from multidrug-resistant (MDR) to pan-drug-resistant (PDR) phenotype. Furthermore, the localization of several resistance genes within mobile genetic elements suggests the species strong capability to acquire resistance factors from clinical biotic and abiotic environments, consistent with previous genome reports on extensively and pan-drug-resistant *A. baumannii* isolates [10].

Virulence determinants associated with biofilm formation and cell–cell adhesion were also identified in the ABS2 genome. These include the quorum sensing system components *AbaI* (autoinducer synthase) and *AbaR* (response regulator), which are essential for biofilm development; the two-component regulatory system *BfmS-BfmR*, involved in signal transduction and biofilm regulation; and the *PgaABCD* operon responsible for the synthesis and export of poly- β -1,6-N-acetylglucosamine (PNAG). Additionally, *CsuA/BABCDE* encodes a chaperone-usher system facilitating pilus assembly and surface attachment, while *OmpA* and *Bap* correspond to outer membrane and biofilm-associated proteins, respectively, Iron uptake and siderophore biosynthesis genes *basABCDGHIJ*, *bauCDEF*, and *entE* were also detected. The presence of these virulence and resistance determinants in the ABS2 genome is consistent with previously characterized *A. baumannii* strains exhibiting strong biofilm-forming ability and multidrug resistance [11].

Genomic analysis of *A. baumannii* ABS2 demonstrated the coexistence of both biofilm-related and antibiotic resistance determinants that support organismal persistence in hospital settings and clinical apparatus. Biofilms serve as a physical and physiological barrier to antibiotics, while resistance genes equip bacteria to withstand antimicrobial exposure. These traits often appear together and may act in concert, since biofilm formation can facilitate the horizontal transfer of antibiotic resistance genes. The selective pressure exerted by extensive antibiotic use further promotes the emergence and persistence of bacterial populations possessing both biofilm-forming capacity and antibiotic resistance determinants [1]. In addition, the enrichment analysis of *A. baumannii* ABS2 genome also suggests that presence of the over-representation of the HhH1 domain, implicated in DNA binding and repair, suggests enhanced genome stability under oxidative or genotoxic stress [44, 45]. The Venn diagram illustrates the protein profiles of ABS2 and the reference strain, indicating 625 shared proteins, with 1338 proteins unique to Sample2 and 307 unique to the reference strain. The increased number of unique proteins in ABS2 indicates genomic plasticity and the acquisition of accessory genes that could improve environmental adaptability, antimicrobial resistance, or virulence potential. This genomic

diversity aligns with earlier studies indicating that *A. baumannii* strains often engage in horizontal gene transfer and genomic rearrangements, which contribute to strain-specific adaptive characteristics [46]. Comparative Reactome pathway enrichment analysis indicated that both the *Acinetobacter baumannii* reference strain and ABS2 showed significant overrepresentation in oxidative stress tolerance pathways. This suggests that both strains have developed enhanced mechanisms for oxidative stress tolerance, which may aid their survival in host immune environments. This finding is consistent with earlier studies that emphasize the significant role of oxidative stress defence in the persistence and virulence of *A. baumannii* [46]. Furthermore, ABS2 exhibited a modest increase in proteins related to the chorismate via the shikimate pathway (19.23% vs. 16.67%), indicating a potential upregulation of aromatic amino acid biosynthesis, which is associated with adaptive metabolic flexibility under nutrient-limited or host-mimicking conditions [47]. An increase in the antimicrobial action and resistance pathway (15.38% vs. 12.50%) indicates a potential expansion of resistance-associated proteins in ABS2, which supports the development of multidrug-resistant phenotypes via metabolic adaptation [6]. The PPI interactions of bioinformatics prediction suggest that the *yajL* gene may be essential for maintaining proteome integrity during stress conditions, especially those induced by oxidative or antibiotic factors. An adaptive characteristic vital for the survival and persistence of *A. baumannii* [32, 48].

Conclusion

The examination of efflux pump functionality and antibiotic susceptibility offers several advantages. This research improves our comprehension of microbial resistance mechanisms, essential for formulating effective therapies against antibiotic-resistant infections, especially those attributed to *A. baumannii*. This methodology enables a comprehensive analysis of the role of biofilms in chronic infections, leading to enhanced strategies for biofilm management. Enrichment analysis elucidates the relationship between antimicrobial resistance and stress tolerance in conjunction with the DNA repair system, which contributes to infection persistence in hosts and survival in harsh environments. Concurrent analysis of efflux pumps and biofilm genes may enable researchers to identify potential targets for novel antimicrobial agents. This integrative study may facilitate the identification of biomarkers for infections linked to biofilms and enhance therapeutic interventions using antibiotics. Additionally, it can improve experimental design by offering a thorough understanding of microbial behaviour in diverse environments.

Limitation of the study

In addition to PCR-based identification of resistance and virulence genes, in this study PCR is used to check gene presence only, and resistance levels or changes in MIC might vary due to the expression level of resistance genes, which were not measured.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11033-026-11791-1>.

Acknowledgements The authors express their sincere gratitude to the VISTAS organization for providing the Vels Research Fellowship (VRF) to support the completion of this research study.

Author contributions All authors made contributions in the conceptualization and writing the manuscript. Sivaranjani Gowrinathan performed the experiments and interpreted the data. Gayathri Gururajan supervised the experiments and drafted the manuscript. Kathireshan A Kaliyaperumal contributed to data analysis and critically revised the manuscript and Senthil Renganathan contributed to the genome analysis. All authors read and approved the manuscript.

Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

Ethics approval The Ethics Committee of VISTAS has approved this study under the reference number VISTAS-SPS/IEC/1/2022/04.

Statement of confirmation The bacterial isolates used in this study was directly procured from hospital as bacterial cultures that were isolated from patient's samples. So, we provided institutional ethical clearance reference number. This study does not involve patients directly hence we did not have informed consent for this study.

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