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Original Article

Exploring Antibiotic Resistance Gene Expression in *Acinetobacter baumannii* Using Microarray Technology

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ABSTRACT

Background: Antibiotic resistance is a significant challenge in healthcare, particularly in nosocomial infections caused by *Acinetobacter baumannii*. Efflux pumps play a crucial role in mediating antibiotic resistance in A. baumannii, yet comprehensive evaluation of these pumps and acquired resistance determinants is lacking. Here, we present the development and validation of an oligonucleotide-based DNA microarray for assessing gene expression of efflux pumps and detecting acquired antibiotic resistance determinants in A. baumannii.

Objective: The primary objective of this study was to develop a robust microarray platform capable of simultaneously assessing the expression of efflux pump genes and detecting acquired resistance determinants in A. baumannii. Additionally, we aimed to validate the microarray's performance using mutants overexpressing or deficient in efflux pumps and single-step mutants obtained on various antibiotics.

Methods: The DNA microarray consisted of probes targeting 78 genes, including 17 efflux systems, 15 resistance determinants, and 19 housekeeping genes. Comparative analysis of mutants, along with quantitative reverse transcriptase PCR validation, was conducted to confirm the microarray's accuracy in detecting efflux pump overexpression.

Results: Validation experiments revealed overexpression of RND efflux pumps *AdeABC* and *AdeIJK* in mutants obtained on gentamicin, cefotaxime, or tetracycline, as well as identification of a novel efflux pump, *AdeFGH*, overexpressed in a mutant exposed to chloramphenicol. Clinical isolates showed overexpression of *AdeABC* and chromosomally encoded cephalosporinase, along with several acquired resistance genes, accounting for the multidrug-resistant phenotype.

Conclusion: The developed microarray demonstrates high sensitivity and specificity in detecting efflux pump expression and acquired resistance determinants in A. baumannii. Its potential utility in identifying antibiotic resistance and novel efflux systems highlights its importance in clinical settings.

Keywords: *Acinetobacter baumannii*, antibiotic resistance, DNA microarray, gene expression profiling

INTRODUCTION

Multidrug-resistant (MDR) strains of *Acinetobacter baumannii* have become increasingly prevalent in recent decades. This opportunistic pathogen causes serious infections, including hospital-acquired pneumonia, as well as bloodstream, urinary tract, and wound infections, raising global concerns (6, 13). Multidrug resistance in A. baumannii is primarily due to either the horizontal transfer of genetic material or mutation of inherent genes. Various resistance mechanisms, such as plasmids, transposons, and integrons are well-documented within Acinetobacter species. Genomic analyses of multiple A. baumannii strains have enriched our understanding of its antibiotic resistance development (1-4). A notable example is the 86-kb resistance island, *AbaR1*, in the *AYE* strain, which harbors approximately 15 antibiotic resistance genes, alongside 10 antiseptic and heavy metal resistance genes. This island is consistently found in a significant number of MDR strains at the same chromosomal location. Resistance is also bolstered by changes in innate functions such as overexpression of the β-lactamases *ADC* and *OXA-51*-like, loss of *CarO* and *Omp33-36* porins

Journal of Health and Rehabilitation Research 2791-156X JHRR

enhancing carbapenem resistance, mutations in the fluoroquinolone targets *GyrA* and *ParC*, and upregulation of efflux systems (3, 5).

Efflux systems, integral to the bacterial membrane, are crucial for cell homeostasis and the expulsion of toxic substances (7,10). They play roles in intercellular communication through quorum sensing and contribute to the pathogenicity of bacteria. Specifically, efflux pumps from the *resistance nodulation cell division (RND)* superfamily are prevalent in gram-negative bacteria and, when overexpressed, impart multidrug resistance. Although the resistance conferred is often moderate, these pumps reduce the intracellular concentration of antibiotics, thereby delaying the emergence of higher-level resistance through drug deactivation or target modification, and effectively synergizing with other resistance mechanisms(1, 12, 15).

Strain	Relevant characteristic(s)
BM4546	CIP 70-10 AdeS$_{T153M}$ spontaneous mutant, MDR
BM4547	CIP 70-10 AdeR$_{P116L}$ spontaneous
BM4579	BM4454 Δ*adeIJK*
BM4651	BM4454 Δ*adeABC*

MATERIAL AND METHODS

The bacterial strains utilized in this study were cultured at 37°C in brain heart infusion broth and agar (Difco Laboratories, Detroit, MI) for growth. Antibiotic susceptibility testing was conducted using disk diffusion on Mueller-Hinton agar (Bio-Rad, Marnes-la-Coquette, France), with MICs determined via the Etest procedure (AB Biodisk, Solna, Sweden). Multidrug-resistant (MDR) mutants, including *BM4665*, and *BM4579*, were obtained from wild-type strains *BM4454* by selection on gradient plates containing gentamicin, cefotaxime, or tetracycline, with resistant colonies further evaluated for multidrug resistance. Genomic DNA extraction from *Acinetobacter baumannii* was performed using established methods, and DNA amplification was carried out using Taq polymerase (MPbio, NY) and a Chipwriter Pro Virtek arrayer (Bio-Rad, Hercules, CA). For microarray analysis, cDNAs were synthesized from 10 µg of total RNA, labeled with Cy3 or Cy5 cyanin (GE Healthcare, Uppsala, Sweden), and hybridized on microarrays. The resulting data underwent script analyses with R software, incorporating normalization and statistical evaluation to identify significantly differentially expressed genes. Quantitative reverse transcriptase (qRT) PCR was conducted for validation, and microarray data were deposited in ArrayExpress (E-MEXP-2254) under MIAME standards.

RESULTS

An oligonucleotide-based DNA microarray was custom-designed to analyze efflux gene expression differences in multidrug-resistant (MDR) A. baumannii mutants and clinical isolates compared to a reference strain. Initially sourced from GenBank due to the absence of an annotated A. baumannii genome, the microarray comprised 205 selected genes. Detailed information is available on Array Express under accession number E-MEXP-2254. The designed probes targeted 47 efflux-related genes from the *AYE* strain, including six RND systems (e.g., *adeABC*, *adeIJK*) (9,14), seven MF systems, and two MATE systems. Probes were also included for genes encoding outer membrane proteins (e.g., HMP-AB, 33-36 kDa protein, CarO) (10), biofilm formation-associated genes (e.g., csu operon) (11), and multiple antibiotic resistance genes from gram-negative bacteria. Additionally, genes conferring resistance to heavy metals (arsenic, mercury) and those involved in genetic element mobility (e.g., integrases, transposases) were incorporated (18, 19). These sequences enabled the detection of mobile genetic element structures carrying antibiotic resistance determinants.

TABLE 1. Primers Sequences used in this Study

Gene	Primer	Sequence (5'–3')	Position	Size (bp)
rpoB	rpoB Rev rpoB For	ATGCCGCCTGAAAAAGTAAC TCCGCACGTAAAGTAGGAAC	1960–1979 2114–2075	152
adeA	adeA For adeA Rev	ATCGCTAACAAAGGCTTGAA CGCCCCCTCAGCTATAGAA	1024–1043 1183–1163	154
adeB	adeB For adeB Rev	CTTGCATTTACGTGTGGTGT GCTTTTCTACTGCACCCAAA	2907–2926 3075–3056	158
adeC	adeC For adeC Rev	TACACATGCGCATATTGGTG CGTAAAATAACTATCCACTCC	1157–1176 1274–1244	127
ampC	ampC For ampC Rev	CAGTAATTCAGAACAGATTGTG GCGCTCTTCATTTGGAATAC	927–947 1110–1091	123
adeS	adeS For adeS Rev	TATGAAAAGTAAGTTAGGAAT TTAGTTATTCATAGAAATTTT	1–19 1073–1053	1,022

Microarray validation involved comparing strain *BM4454*, which overexpresses the *AdeABC* efflux system, with mutants *BM4579*, *BM4651, and BM4652*, where *adeIJK*, *adeABC*, and both systems were inactivated, respectively. Analysis revealed that *adeI* and *adeJ* genes were not expressed in *BM4579*, confirming the inactivation. Similarly, in *BM4651* (*ΔadeABC*), *adeA* and *adeB* were confirmed inactive, with the *aac(3)-IVa* cassette expressed. In *BM4652* (*ΔadeABC ΔadeIJK*), *adeA*, *adeB, adeI,* and *adeJ* were inactive, and *aac(3)-IVa* and *adeC* were overexpressed (18,20). Validation was also conducted using *AdeABC*-overexpressing mutants *BM4546* and *BM4547*, revealing differential *adeA* and

Journal of Health and Rehabilitation Research 2791-156X JHRR

adeB expression levels, particularly in BM4546 with higher *adeB* mRNA amounts. This study confirmed regulatory control by the AdeRS two-component system, showing distinct expression patterns in mutant strains (21,23).

TABLE 2. Comparative Analysis of Gene Expression in Isogenic Strains of A. baumannii

"NS" denotes not statistically significant, and "NA" signifies not applicable. "Not statistically significant by the microarray but confirmed by qRT-PCR."

Strain		Change (fold)		
Parent	Mutant	*adeA*	*adeB*	*adeC*
BM4454	BM4579	NS	NS	NS
	BM4651	—4.21	—3.25	1.84
	BM4652	—1.17[b]	—1.50	1.38[b]
CIP70-10	BM4546	2.17	2.74	NS
	BM4547	NS	NS	NS
BM4587	BM4665	1.79	3.04	2.09
	BM4666	NS	NS	NS
BM4667	BM4668	NS	NS	NS

Mutant strain BM4665, derived from susceptible clinical isolate BM4587, exhibited resistance to several antibiotics, with overexpression of the *adeABC* operon (1.8- to 3-fold increase) confirmed by qRT-PCR. Mutations in adeRS likely contributed to this overexpression. Mutants BM4666 and BM4668, derived from BM4587 and BM4667, respectively, showed distinct resistance profiles and overexpression of *adeI* and *adeJ*, indicating increased expression of the *adeIJK* gene set. Another mutant, *BM4652* (*ΔadeABC ΔadeIJK*), displayed overexpression of the AdeFGH efflux system, potentially contributing to its multidrug resistance phenotype(19, 22).

TABLE 3. AST profile of *A. baumannii*
MIC (µg/ml)

	TIC	TIM	CAZ	CTX	FEP	ATM	IPM	CHL	TET	MIN	TIG
BM4454	8	12	6	4	3	16	0.25	>256	32	0.75	3
BM4587	6	8	4	3	0.75	12	0.19	96	1.5	0.064	0.094
AYE	>256	>256	>256	>256	>256	>256	1	>256	64	0.5	1.5
BM4675	>256	>256	>256	>256	8	32	2	>256	32	8	1.5
BM4676	>256	>256	>256	32	32	32	>32	128	12	1	1.5

BM4676 exhibited high-level resistance to imipenem, β-lactams, chloramphenicol, fluoroquinolones, and tetracycline, along with decreased susceptibility to minocycline and tigecycline. It showed moderate-level resistance to netilmicin and high resistance to other aminoglycosides. Transcriptome analysis indicated overexpression of chromosomal genes *adc* and *adeABC*, along with weak overexpression of *adeI* and *adeJ*. Detection of OXA-23 carbapenemase and aminoglycoside resistance genes, facilitated by the microarray, explained most of BM4676's multidrug resistance. Additionally, the microarray identified the presence of transposase genes associated with the *AbaR1* resistance island, impacting membrane permeability and antibiotic susceptibility. This technology is valuable for quantifying gene expression and detecting antibiotic resistance mechanisms in A. baumannii clinical isolates.

CONCLUSION

The newly developed oligonucleotide-based DNA microarray for *Acinetobacter baumannii* represents a significant advance in our ability to understand and combat antibiotic resistance. It accurately assesses efflux pump expression and identifies acquired resistance determinants, proving essential for managing multidrug resistance in clinical settings. Validation with mutants confirms its reliability, and its potential in identifying novel resistance mechanisms highlights its value in both research and clinical diagnostics. As antibiotic resistance continues to pose a serious challenge in healthcare, this microarray offers a promising tool for early detection and targeted treatment strategies, ultimately enhancing patient outcomes and informing antibiotic stewardship.

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Journal of Health and Rehabilitation Research 2791-156X JHRR

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