Molecular Analyses of Biofilm-Producing Clinical *Acinetobacter baumannii* Isolates from a South Indian Tertiary Care Hospital

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Highlights of the Study

- The emergence of colistin-resistant *Acinetobacter baumannii* led to analysis of clinically isolated *A. baumannii*.
- Isolates collected over 6 months were analysed for biofilm production, genes associated with antibiotic resistance and virulence, and mobile genetic elements (MGEs).
- Strict implementation of infection control guidelines is critical to combat the spread of antimicrobial resistance.

Keywords

A. baumannii · Tn1213 · AbaR (*Acinetobacter baumannii* antibiotic resistance) island · Biofilm

Abstract

Objectives: The aim of the study was to determine the presence of antimicrobial-resistance (AMR) genes, virulence genes, and mobile genetic elements (MGEs) in 14 biofilm-producing clinical isolates of *Acinetobacter baumannii*. **Materials and Methods:** PCR amplification was performed to analyse the prevalence of genes associated with antibiotic resistance (extended-spectrum β -lactamases [ESBLs] and metallo- β -lactamases [MBLs]), virulence factors, MGEs (class 1 integron, Tn1213, and *A. baumannii* antibiotic resistance

[AbaR]), and *comM* among the study isolates. Random amplified polymorphic DNA (RAPD) PCR was then deployed to understand their phylogenetic relationship. All the isolates were investigated for biofilm production. **Results:** Two isolates were antibiotic-sensitive (AS), 3 were multi-drug-resistant (MDR), and the remaining 9 were extensively drug-resistant (XDR). The majority of the isolates were found to be positive for biofilm production and were sensitive against tetracycline and colistin only. Ab14 and Ab11 were found to be resistant to minocycline and colistin, respectively. *bla*_{TEM}, *bla*_{OXA}, *bla*_{NDM}, *bla*_{VIM}, *bla*_{SIM}, and *bla*_{PER-1}; class 1 integron; composite transposon Tn1213; AbaR island, and virulence factor genes were detected among the isolates. These pathogens were found to have originated from multiple clonal lineages. **Conclusion:** Biofilm-producing *A. baumannii* with

multiple virulence and AMR genes pose serious clinical challenges. The presence of MGEs further compounds the situation as these isolates serve as potential reservoirs of AMR and virulence genes. Together with their capacity for natural competence, *A. baumannii*, if left unchecked, will lead to the spread of resistance determinants to previously sensitive bacteria and may aid in the emergence of untreatable pan-drug-resistant phenotypes.

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Introduction

Acinetobacter baumannii causes a variety of community-acquired and nosocomial infections of the cerebrospinal fluid, lungs, wounds, urinary tract, skin, soft tissue, and bloodstream, with high rates of morbidity and mortality [1–3]. *A. baumannii* topped the WHO (World Health Organisation) list of 12 bacteria for which new drugs are urgently required [4]. Irrational use of antibiotics and selective pressure has led to *A. baumannii* emerging as an antibiotic-resistant pathogen in both hospital and environmental settings. An increased prevalence of multi-drug-resistant (MDR) and extensively drug-resistant (XDR) strains of *A. baumannii* have been observed in large numbers over the past 2 decades [5]. These pathogens are found to persist over extended periods of time on inanimate surfaces under a wide range of environmental conditions, largely due to their capacity to produce strong biofilms [6]. Scant information is available with regard to the clonal relatedness and diversity of *A. baumannii* strains on the Indian subcontinent. Genomic analysis of MDR *A. baumannii* has indicated the presence of integrons, transposons, and other mobile genetic elements (MGEs) such as *A. baumannii* antibiotic resistance (AbaR) islands. Acquisition of these resistance determinants has been attributed to the potential for horizontal gene transfer among *A. baumannii* isolates [7, 8]. The ATPase-encoding gene *comM* (competence protein) is split by the incoming AbaR island, leading to the formation of 2 segments, i.e., *comM*-AbaR and AbaR-*comM* [9]. Tn1213 is a composite transposon made up of up to two distinct insertion sequences, ISPa12 and ISPa13 [10]. An extended-spectrum β -lactamase (ESBL), *bla*_{PER-1}, is generally ferried by this transposon across the genomes [11]. Furthermore, these pathogenic bacteria are reported to harbor several virulence factors that help in quorum sensing and regulation, i.e., adherence, drug efflux, biofilm formation, and serum resistance. They also help the pathogen to colonize the host, evade or suppress the

host's immune response, obtain essential nutrients and growth factors from the host, and gain entry or exit from the host.

In this study, colistin-resistant (MDR) *A. baumannii* was isolated from the blood collected at central line of a patient operated for coronary artery bypass at the Sri Sathya Sai Institute of Higher Medical Sciences, Prasanthigram (SSSIHMS-PG). This prompted the Infection Control Board to investigate *A. baumannii* infections over a period of 6 months, from October 2015 to March 2016. Here, a total of 14 clinically isolated *A. baumannii* (2 antibiotic-sensitive [AS], 3 MDR, and 9 XDR isolates) were analysed for the prevalence of virulence factors and antibiotic resistance genes. The isolates were then screened for the presence of composite transposon Tn1213, *bla*_{PER-1}, and the host gene *comM* to understand their genetic context. All the isolates were investigated for their capacity to produce biofilms.

Materials and Methods

Setting

The study included 14 patients with cardiac, urological, orthopaedic, and general medicine related problems, who underwent treatment at the SSSIHMS-PG. Samples included 9 isolates from pus, 2 from urine, and 1 each from the pleural fluid, blood, and endotracheal tube (Table 1). All samples were cultured at the Department of Microbiology, SSSIHMS-PG. Only one isolate from each patient was included in the study.

Background

A 59-year-old male patient with a history of left main coronary artery disease underwent an operation in December 2015. Due to low ejection fraction, he was admitted to the intensive care unit post-operatively and required prolonged ventilator and inotropic support. In January 2016, blood culture from a central line catheter revealed the presence of colistin-resistant, MDR *A. baumannii*. To further understand the prevalence of *A. baumannii* among patients being treated at this centre, all *A. baumannii* infections reported over a 6-month period (October 2015 to March 2016) were analysed.

Identification and Antibiotic Susceptibility Testing

Organism identification and antibiotic susceptibility testing (AST) of the 14 *A. baumannii* isolates was performed using VITEK 2.0 compact system and GN-281 AST cards (Table 2). Interpretation of the AST was done as per CLSI (Clinical Laboratory Standards Institute) guidelines [12]. Furthermore, glycerol stocks of the pure cultures were made and stored at -80°C for future use. Based on the antibiotic resistance patterns, the 14 study isolates were categorized as AS, MDR, or XDR.

Whole-Genomic DNA Extraction and Quantification

Whole-genomic DNA from all the isolates was extracted from 3 mL of overnight LB broth culture by following the manufac-

Table 1. Sources of sample collection

Sample ID	Age	Gender	Source	Chief clinical complaint	Date of isolation
Ab 1	9 months	male	pus	congenital cyanotic heart disease	October 3, 2015
Ab 2	65 years	male	urine	urological dysfunction	October 20, 2015
Ab 3	20 years	female	pus	wound infection	October 21, 2015
Ab 4	10 months	male	pleural fluid	congenital cyanotic heart disease	October 28, 2015
Ab 5	22 years	female	pus	wound infection	November 7, 2015
Ab 6	32 years	male	pus	fracture wound	November 20, 2015
Ab 7	50 years	male	pus	fracture wound	November 18, 2015
Ab 8	69 years	female	pus	fracture wound	December 14, 2015
Ab 11	59 years	male	blood	coronary artery disease	January 10, 2016
Ab 12	8 years	female	urine	fracture wound	February 5, 2016
Ab 13	25 years	male	pus	fracture wound	February 16, 2016
Ab 14	59 years	male	pus	trauma wound	February 25, 2016
Ab 15	57 years	male	pus	chronic osteomyelitis; with pathological subtrochanteric fracture	March 15, 2016
Ab 16	56 years	male	endotracheal tube	chronic rheumatic heart disease	March 18, 2016

turer's protocol (Machery-Nagel NucleoSpin-Tissue DNA extraction kit). Quantification of the DNA was done by NanoDrop, while the quality of DNA was assessed by agarose (0.8%) gel electrophoresis.

PCR Screening

Gene sequences of virulence factors were obtained from the virulence factor database (VFDB- <http://www.mgc.ac.cn/VFs/>) and specific primers were designed for 10 virulence factors (*ompA*, *bfmR*, *abaI*, *abaR*, *ptkA*, *plc*, *pld*, *hemO*, *adeF*, and *pbpG*). Screening of virulence genes, AMR genes, AbaR islands (with or without disruption of the *comM* gene), and composite transposon Tn1213 was performed as described previously [11, 13], or standardized in this study. Three primers were used to amplify (a) intact *comM* without any insertion, (b) initial transitional region *comM*-AbaR, and (c) final transitional region AbaR-*comM*. Primer details and PCR conditions are tabulated in online supplementary Tables 1 and 2 (see www.karger.com/doi/10.1159/000508461 for all online suppl. material), respectively. PCR was carried out using a final concentration of 0.5 μ M of each primer in a 20- μ L reaction. TaKaRa's Sap- phireAmp 2X PCR master mix was used for all the PCR amplifications.

Random Amplified Polymorphic DNA-Based Phylogeny

The clonal relationship among the 14 study isolates was studied. Twelve different random amplified polymorphic DNA (RAPD) primers (online suppl. Table 1) were used for PCR amplification, and the banding pattern from the primer which produced bands in all the isolates was further analysed by SYNGENE GeneTools software. A dendrogram was generated using the unweighted pair group method with arithmetic mean (UPGMA).

Biofilm Formation

Biofilm formation was investigated. Briefly, 100- μ L aliquots of bacterial culture (OD₆₀₀ of 0.1) were inoculated in 96-well round-bottom plates and incubated without shaking for 48 h at 37 °C. Following incubation, the plates were washed 3 times using 200 μ L

ddH₂O, and then stained with 0.1% crystal violet (125 μ L/well) for 10 min at room temperature. The plates were then washed twice with 200 μ L ddH₂O to remove excess crystal violet and dried overnight at room temperature. Solubilisation of the crystal violet was performed by adding 200 μ L of 30% acetic acid per well for 10 min at room temperature. Finally, 125 μ L of the solubilised mixture was transferred to a fresh flat-bottom 96-well plate and the biofilm production was quantified (OD₅₉₅) using a UV-Vis spectrophotometer. All experiments were conducted in triplicate [14].

Results

Fourteen *A. baumannii* isolates were collected from pus, blood, pleural fluid, endotracheal tube, and urine samples from 4 female and 10 male patients (Table 1) who underwent treatment at the SSSIHMS-PG. Nine of these isolates were obtained from patients with fractures (wound infections), 4 were isolated from patients with cardiac ailments, and 1 was from a patient with urological dysfunction. The age of the patients ranged from 9 months to 69 years. Thirteen of the isolates were from patients with community-acquired infections and 1 patient was found to have post-operative/nosocomial infection with colistin resistance (Ab11).

VITEK 2-based AST indicated that 71.4% (10 isolates) were resistant to piperacillin/tazobactam; 78.57% (11 isolates) were resistant to ceftazidime and cefepime; 57.14% (8 isolates) were resistant to cefaperazone/sulbactam and levofloxacin; 64.28% (9 isolates) were resistant to doripenem, imipenem, ciprofloxacin and trimethoprim-/sulfamethoxazole; and 50% (7 isolates) were resistant to

Table 2. Antibiotic resistance profile of 14 isolates

[illegible]

Table 3. PCR amplification results for genes associated with antibiotic resistance, transposons, resistance islands, and virulence factors

Isolate	AMR genes										comM gene										Virulence genes															
	bla _{CTX-M}	bla _{TEM}	bla _{SHV}	bla _{OXA}	bla _{KPC}	bla _{NDM}	bla _{IMP}	bla _{VIM}	bla _{SIM}	bla _{SPM}	Int1	IncS	sufI	qacE1	bla _{PER-1}	mcr-1	mcr-2	mcr-3	mcr-4	mcr-5	Tn1213	comM	comM-	AbaR	AbaR-comM	ompA	adeF	pbpG	plc	pid	hemO	abaI	abar	bfnR	pitA	
AB1	N	N	N	N	N	N	N	N	N	N	Y	N	Y	Y	N	N	N	N	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	Y	
AB2	N	Y	N	N	N	N	N	N	N	N	Y	N	Y	Y	N	N	N	N	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	Y	
AB3	N	N	N	Y	N	N	Y	N	N	N	Y	N	Y	N	Y	N	N	N	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	
AB4	N	Y	N	N	N	Y	N	N	N	N	Y	N	Y	Y	Y	N	N	N	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	
AB5	N	Y	N	N	N	Y	N	N	N	N	Y	N	Y	Y	Y	N	N	N	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	
AB6	N	Y	N	N	N	Y	N	N	N	N	Y	N	Y	Y	Y	N	N	N	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	Y	
AB7	N	N	N	N	N	Y	N	N	N	N	Y	N	Y	Y	Y	N	N	N	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	
AB8	N	Y	N	Y	N	N	N	N	Y	N	Y	N	Y	Y	Y	N	N	N	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	
AB11	N	Y	N	N	N	N	N	N	N	N	Y	N	Y	Y	Y	N	N	N	N	N	N	NB	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	
AB12	N	N	N	N	N	Y	N	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	
AB13	N	Y	N	N	N	Y	N	Y	N	N	Y	N	Y	N	Y	N	N	N	N	N	N	N	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y	Y	Y	
AB14	N	Y	N	N	N	N	N	N	N	N	Y	N	Y	Y	Y	N	N	N	N	N	N	N	NB	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y
AB15	N	Y	N	N	N	N	N	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	NB	NB	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
AB16	N	N	N	N	N	N	Y	N	N	N	Y	N	Y	Y	Y	N	N	N	N	N	N	Y	NB	NB	Y	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y

AMR, antimicrobial-resistant; N, not detected; Y, amplification positive; NB, multiple bands/differe bands.

Fig. 1. Biofilm development (at 48 h) on microtitre plate wells by clinical isolates of *Acinetobacter baumannii*.

gentamicin. Significantly, all isolates were found to be resistant to monobactam (Aztreonam) but sensitive to tigecycline (Table 2). Importantly, Ab14 and Ab11 were resistant to minocycline and colistin, respectively. Based on the antibiotic sensitivity patterns, the 14 isolates were categorized as AS (Ab15 and Ab16), MDR (Ab8, Ab11, and Ab13), and XDR (Ab1, Ab2, Ab3, Ab4, Ab5, Ab6, Ab7, Ab12 and Ab14).

PCR screening of the 14 isolates suggested that they all contain at least 1 category of AMR-related gene (Table 3); 64.28% were positive for *bla*_{TEM}, 14.28% (2 isolates) were positive for *bla*_{OXA} and *bla*_{VIM}; 42.8% (6 isolates) were positive for *bla*_{NDM}; only 1 isolate was positive for *bla*_{SIM}, while *bla*_{PER-1} was found in all isolates. *sul1* was present in 85.7% (12) of the isolates and *qacE1* in 71.42% (10) of the isolates. All the isolates were found to possess the *Int11* gene. None of the mobilizable colistin-resistance genes (*mcr-1–mcr-5*) were detected in the study isolates including Ab11.

Our investigations revealed the presence of *AbaR* islands in several of the study isolates. Screening of the isolates for *AbaR* islands and the *comM* ATPase gene revealed the presence of intact *comM* gene in 2 (Ab8 and Ab16), while the presence of *AbaR* was confirmed in 10

of the study isolates. In the 4 remaining isolates (Ab8, Ab14, Ab15, and Ab16), our results did not confirm the presence or absence of *AbaR*. Interestingly, *Tn1213*, a composite transposon, which is known to carry *bla*_{PER-1} [10] was not found in any of the study isolates but all of them were found to possess *bla*_{PER-1} (online suppl. Fig. 1–4).

PCR analyses for the presence of 10 selected virulence factor genes revealed that all 14 isolates were positive for *ompA*, *adeF*, *abaI*, *bfpR*, *abaR*, *plc*, and *ptkA* virulence factors. *pbpG* was present in 13 (92.8%) of the study isolates, *pld* in 11 (78.57%), and *hemO* in 10 (71.4%). The presence of the *bfpR* gene, which is involved in the production of biofilm, in all 14 isolates, suggested that the *A. baumannii* under study may be capable of biofilm production. Our investigations revealed that majority of the isolates (71.4%) are strong biofilm producers (Fig. 1; online suppl. Fig. 5). Interestingly, the biofilm production potential of the AS Ab15 and Ab16 isolates was found to be higher than in the Ab1, Ab2, and Ab3 isolates which were characterized as XDR.

Phylogenetic analysis of the 14 *A. baumannii* isolates under study by RAPD PCR suggested that they are unrelated and may have different clonal origins. In this regard,

Fig. 2. RAPD profile of 14 *A. baumannii* isolates using the OPA-3 primer.

12 different primers were used to identify an appropriate primer for *A. baumannii* isolated from the Indian sub-continent. Primer OPA-3 was found to amplify and produce good banding patterns against all the tested isolates (Fig. 2). Phylogenetically, Ab11 (colistin-resistant *A. baumannii*) was not related to the other study isolates. However, its banding pattern indicated some relation to Ab15, which is phenotypically AS.

Discussion

Infections associated with *A. baumannii* are difficult to treat. With the increased emergence of MDR, XDR, and pan-drug-resistant (PDR) variants of this pathogen, limited therapeutic options are available to the treating clinicians. With its high population density and the easy access to antibiotics through their sale over the counter, the Indian subcontinent is a fertile ground for the spread of resistance determinants and the emergence of highly resistant bacterial phenotypes.

In this study, 12 isolates (85.7%) were found to be antibiotic-resistant and only 2 were sensitive against most of the tested antibiotics. Phylogenetic analyses of RAPD suggested that the study isolates do not have a common origin. Our study findings correlate with previous reports on increased resistance against the fluoroquinolone [15] and β -lactam [16–18] classes of antibiotics among *A. baumannii* isolates; this rules out their use as a first-line drug. We found that *bla*_{TEM} is the most prevalent ESBL among *A. baumannii* isolates, although previous reports have indicated that *bla*_{CTX-M} is the pre-

dominant ESBL gene [19, 20]. Similar findings were observed in our previous report on carbapenem-resistant Enterobacteriaceae [8]. This study suggests that *bla*_{SHV} is not a commonly encountered ESBL gene among *A. baumannii* strains on the Indian subcontinent. These results indicate the existence of variations in terms of prevalence and ESBL types in different geographical regions and demonstrate the diversity in the bacterial gene pool.

Generally, imipenem (carbapenems) and amikacin (aminoglycosides) are found to be the most active antimicrobials against *A. baumannii* [21]. However, 64% of the study isolates were found to be resistant to doripenem and imipenem antibiotics. This was confirmed by the presence of at least one carbapenemase/MBL (metallo- β -lactamase) genes in these isolates. This is in line with recent reports on increasing carbapenem resistance among bacteria [22]. *bla*_{OXA} was identified in 2 isolates, *bla*_{NDM} in 6, *bla*_{VIM} in 3, and *bla*_{SIM} in 1. As described previously, *bla*_{GIM} and *bla*_{SPM} were not identified in the study isolates. Our data represents a minor fraction of a hospital's meta-data and may not be representative of the current scenario. The presence of colistin resistance in the nosocomial isolate Ab11 reveals the complexity in dealing with pathogens resistant to the dwindling number of front-line drugs. It is imperative that these antibiotics of last resort are used judiciously before it is too late. The absence of any of the tested *mcr* genes [23] in the isolate Ab11 suggests that other mechanisms such as efflux pumps [24] and those which are yet to be described may have contributed to its emergence as colistin-resistant.

The AbaR island integrated into *comM* [9] has been detected in these strains. We found that 12 isolates had AbaR integrated into the *comM* gene, and 2 had intact *comM* gene in addition to AbaR integrated into the *comM* cassette. This indicates that there might be >1 copy of *comM* gene in these isolates and that 1 remained intact while the other was disrupted due to the integration of AbaR. Tn1213 is a composite transposon, which is inserted specifically between ISPa12 and ISPa13 [10]. Although our results suggested that Tn1213 was not present, *bla*_{PER-1}, commonly associated with Tn1213, was found to be prevalent in all 14 *A. baumannii* isolates, suggesting that multiple mechanisms are involved in the dissemination of *bla*_{PER-1}. Many of the class 1 integron genes identified from clinical settings reveal modifications to their ancestral composition including the incorporation of sulfonamide resistance gene (*sul1*) and/or the deletion of *qacE1* (a determinant of quaternary ammonium compound resistance). In our study, all 14 isolates were positive for the *Int11* gene, 12 were positive for *sul1*, and 10 were positive for *qacE1*. The presence of *qacE1* among the study isolates, a gene commonly associated with the class 1 integron, which provides resistance to quaternary ammonium compounds, correlates with the increased use of detergents and disinfectants in hospital settings. The rapid dissemination of MGEs possessing antibiotic resistance and virulence factor gene cassettes may have been aided by the increase in global travel [25]. This is exacerbated by the capacity of the pathogens to produce biofilms, which poses a significant clinical challenge in the treatment of hospitalized patients.

Conclusion

This study presents the molecular characteristics of clinically isolated, pathogenic, and antibiotic-resistant *A. baumannii* from a tertiary care hospital in South India. The prevalence of MDR and XDR pathogens including those resistant to colistin, although limited in number, is an alarming situation that needs urgent and drastic interventions. Indiscriminate use of antibiotics may be a very strong selection pressure for the maintenance and spread of several resistance determinants among the clinical isolates. The presence of MGEs further compounds the already deteriorating situation due to the lack of availability of newer classes of antibiotics and therapeutic agents. Implementation of the current national and international guidelines in combatting these dangerous pathogens assumes critical proportions due to their extreme pathogenic potential, including those which were found to be AS. In this regard, devising and incorporating appropriate infection control measures, together with robust antibiotic stewardship programs, is recommended.

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Conflict of Interest Statement

There were no conflicts of interest.

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