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Original Article

Genetic Characterization of Extensive Drug Resistant *Acinetobacter Baumannii*: an Appalling Impediment

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Abstract

Introduction: *Acinetobacter baumannii* infections are a growing public-health concern. The bacterium's potentiality to acquire resistance to a number of commonly used antibiotics has turned it into a formidable pathogen.

Aims: Molecular characterization of extensive drug resistant (XDR) typing of *A. baumannii* clinical isolates by polymerase chain reaction.

Materials and methods: Thirty XDR *A. baumannii* were investigated for the presence of genes encoding carbapenemase resistance, biofilm capacity, autoinducer synthase, virulence and surface motility by polymerase chain reaction (PCR). Later, the isolates were typed by plasmid-based replicon (Rep) (PBRT) and trilocus sequence typing.

Results: All 30 XDR *A. baumannii* strains displayed genes related to surface motility, autoinducer synthase, virulence determinant, biofilm related genes except PER, and *bap*, the frequency of which was 83.3% and 76.6%, respectively. Analysis of *rep* genes showed highest frequency of *rep6* and *rep2* genes, with frequency of 75% and 65%, respectively. All XDR *A. baumannii* strains belonged to SG I (European clone II) group.

Conclusions: Our results show the extraordinary plasticity of XDR *A. baumannii* and suggest that the strains have gained endemicity in our hospital, which could be a great concern in the near future.

Keywords

Acinetobacter baumannii, autoinducer synthase, biofilm, carbapenem-hydrolyzing class D β-lactamases, extensive drug-resistance, replicase typing, trilocus sequence typing, virulence

INTRODUCTION

Acinetobacter baumannii, famed as an "old friend-new enemy"[1], has emerged as a successful pathogen causing a broad array of clinical infections in hospital and community acquired settings. The organism possesses intrinsic and acquired resistance to a number of commonly used antibiotics and is thus gradually bequeathing intra and inter-hospital setting.[2] Nevertheless, antibiotic resistance rates can vary according to the country, the individual hospital, and may even depend on biological, epidemiological or methodical factors.[3] As the organism has gained hold of the antibiotic resistance, it is not uncommon to find multidrug-resistant (MDR, resistance to at least three classes of antimicrobials), extensively drug-resistant (XDR, MDR plus resistance to carbapenems), and pan-drug-resistant (PDR, XDR plus resistance to polymyxins) nosocomial isolates that are hard to treat with the currently available drugs.[4] Amongst all these, according to World Health Organization (WHO) carbapenem-resistant *A. baumannii* (CRAB) strains have emerged as one of the most concerning antibiotic-resistant pathogens among other gram-negative bacteria.[5]

Carbapenem-hydrolyzing class D β-lactamases (CHDLs) are determinants of carbapenem resistance in *A. baumannii*. Four major plasmid encoded CHDLs genes including $bla_{OXA-24/40}$, bla_{OXA-23}, bla_{OXA-58}, and $bla_{OXA-51-like}$ have been documented globally.[6] Amongst them, strains carrying bla_{OXA-58} stand out as the most common type from Europe, Argentina, Australia, the United States and many Asian countries.[7]

One of the major factors contributing to drug resistance in *A. baumannii* associated infections is its biofilm development capacity.[8] Quorum sensing (QS) (autoinducer-receptor mechanism) plays a role in biofilm formation in *Acinetobacter baumannii* associated infections, though its role in the regulation of other virulence factors is yet to be established.[9] Several published studies have explored the relationship between biofilm and antibiotic resistance in *A. baumannii.*[10-13] In this regard, *A. baumannii* is among the leading nosocomial pathogens with a capacity to colonize venous catheters (CVCs) and cause lower respiratory tract infections (due to contaminated ventilators).[14] Bacteria in the biofilm structure are enclosed in a matrix that increases drug resistance and causes chronic and persistent diseases that are difficult to treat.[15,16] Research conducted on biofilm-associated operon in *A. baumannii* suggested *CsuA/BABCDE*-mediated pili formation, which plays a role in the initial steps of biofilm by allowing bacterial cells to adhere to abiotic surfaces resulting in the initiation of microcolony formation that precedes the full development of biofilm structures. *CsuE* codes for the tip adhesion and its inactivation result in the abolition of pili production as well as biofilm formation.[17] The expression of this operon has been found to be regulated by a two-component system comprising sensor kinase encoded by *bfmS* and a response regulator *bfmR*.[18] BfmSR controls the production of capsular exopolysaccharides as well as pilus assembly, and consequently, cell attachment and biofilm formation.[19] *A. baumannii* also contains *pgaABCD* locus that encodes a protein which synthesizes cell-associated poly-β-(1-6)-N-acetyl glucosamine (PNAG).[20] In a variety of gram-negative bacteria, it has been demonstrated that biofilm development may depend on *N*-acyl-homoserine lactone (AHL) signalling molecules.[21] Mass spectrometry has identified AHL signals directed by protein AbaI. The *abaI* gene is activated in a positive-feedback loop by an AbaI-dependent AHL signal(s).[21] In addition, a homolog of a staphylococcal biofilm-associated protein (Bap) has been characterized in *A. baumannii*, where it appears to act as an extracellular adhesin and play a key role in biofilm production in *A. baumannii*.[22] *blaPER-1* and *Bap*, in addition to being involved in biofilm formation, are also involved in the bacterial attachment to human epithelial cells and abiotic surfaces.[23] The formation of pellicles, a specific form of biofilm, occurs at the air-liquid interface and is distinct from submerged biofilms. A correlation between surface associated motility and pellicle biofilm formation has been described for *A. baumannii*.[24]

The organism encodes a diverse range of secretion systems. The type I secretion system (T1SS) is a tripartite system, delivering proteins from the cytosol to the extracellular environment. Interestingly, the activity of the T1SS was shown to have a direct impact on the type VI secretion system (T6SS), suggestive of cross talk between these systems.[25] Among various virulence factors possessed by *A. baumannii*, outer membrane protein A (OmpA, previously Omp38) is the most abundant *A. baumannii* OMP and one of the most well-characterized virulence factors.[26]

Despite the progress in the study of antibiotic resistance mechanisms in *A. baumannii*, a more recondite work is knowledge about the genetic factors that have driven the recent evolution of *A. baumannii* toward multidrug resistance.[27] Sequence analysis of plasmid replicons corresponding to *A. baumannii* clinical strains has revealed many differences with those from other bacterial species, strongly suggesting that *A. baumannii* contains its own plasmid types.[28] Research on the genome content of *A. baumannii* species reveals the specificity of its plasmids and thus, plasmid typing of *A. baumannii* according to their replicase (Rep) proteins was suggested as an effective tool.[28] Plasmid-based replicon (Rep) (PBRT) typing scheme furnished that there are 19 homology groups (GR1–GR19) based on their nucleotide sequence similarities.[27,29] PBRT method is particularly useful when investigating specific features such as an antibiotic resistance gene. Intriguingly, it will be a very useful method to detect and study these plasmids and to further understand the evolution of resistance.[27]

A multilocus sequence-typing scheme or the "trilocus sequence-based typing" (3LST), based on housekeeping genes, for *A. baumannii* examined the sequence variation within three genes likely to be under selective pressure. This approach, which has the potential for greater discrimination, comprises of three genes: *ompA*, *csuE* and $bla_{OXA-51-like}$.

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Outer-membrane protein A (encoded by *ompA*) is a porin found to induce apoptosis of epithelial cells. The *csuE* gene codes for part of a pilus assembly system and is essential for biofilm formation.[30] The $bla_{OXA\text{-}51\text{-}like}$ gene codes for the intrinsic carbapenemase found in *A. baumannii*.[30,31] The 3LST-based multiplex-PCR assay's purpose was to rapidly assign isolates to profile groups (G), consisting of G1, corresponding to ICII; G2, to ICI; and G3, to ICIII.[30] Subsequently, several studies using this PCR-based typing technique reported the identification of new amplicon combinations, in addition to those expected for ICI-III clones.[31]

AIM

The present study determined I) the correlation between the ability of *A. baumannii* for biofilm formation and distribution of biofilm related genes, and II) carbapenem resistance genotypes in the XDR *A. baumannii* clinical isolates. Furthermore, we applied PBRT and 3LST schemes to type the isolates according to their replicase and housekeeping genes, respectively.

MATERIALS AND METHODS

Bacterial isolates

We performed this study on 30 XDR *A. baumannii* strains isolated from clinical specimens obtained from various patients admitted in Sina Hospital, a University based Teaching and Research Center (Tabriz, Iran) as a routine bacteriological procedure. The *A. baumannii* isolates were initially identified by conventional phenotypic methods[32] and later confirmed genotypically ($bla_{Oxa\text{-}51}$ and *rpo* genes).[33,34] *Acinetobacter baumannii* ATCC19606 was used as a standard strain.

Antimicrobial susceptibility testing

The antibiotic susceptibility of *A. baumannii* isolates was based on the disk diffusion and agar dilution (minimum inhibitory concentration, MIC) methods. The disk diffusion was done by inoculating bacterial culture (turbidity matched equivalent to 0.5 McFarland standard) onto Mueller Hinton agar plate, according to Clinical Laboratory Standard Institute (CLSI) guidelines.[35] Antibiotics used to assess the susceptibility of *A. baumannii* isolates were: ceftazidime (30 μg), cefepime (30 μg), sulfamethoxazole/trimethoprim (1.25/23.75 μg), amikacin (30 μg), gentamicin (10 μg), ceftriaxone (30 μg), ciprofloxacin (5 μg), ampicillin-sulbactam (10 μg/10 μg), imipenem (10 μg), and meropenem (10 μg) (Mast Group Co, UK). The interpretative zones were measured and interpreted as per CLSI recommendations.[35] The MIC of colistin and tigecycline (Sigma-Aldrich, St Louis, MO, USA) was determined by agar dilution by preparing serial dilutions (from 0.25 to 256 μg/mL) of above mentioned antibiotics as per CLSI guidelines.[35] Extensive-drug resistance (XDR) was defined in this analysis as resistance to all following drug classes except colistin and tigecycline.[36] Extended-spectrum cephalosporins (ceftazidime and cefepime), aminoglycosides (amikacin and gentamicin), folate pathway inhibitors (sulfamethoxazole/trimethoprim), quinolones (ciprofloxacin) and carbapenems (imipenem and meropenem). *Escherichia coli* ATCC 25922 was used as a quality control strain. There are no CLSI *Enterobactericeae* MIC breakpoints for tigecycline, so the FDA breakpoints for susceptible (MIC ≤ 2 mg/liter), intermediate (4 mg/liter), and resistant (MIC ≥ 8 mg/liter) were used to categorize tigecycline susceptibility. Tigecycline showed considerable, though not consistent, antimicrobial activity against MDR (including carbapenem-resistant) *A. baumannii* isolates.[37] A research study conducted concluded that a breakpoint zone diameter of ≥16/≤12 mm to define susceptibility/resistance, respectively, instead of those proposed by the U.S. Food and Drug Administration (FDA) for *Enterobactericeae* organisms (≥19/≤14 mm, respectively), reduces the intermethod minor errors to an acceptable level (only 9.7% instead of 23.3%, with the FDA breakpoints proposed).[38]

Detection of *blaOXA* genes

The bacterial genomic DNA was extracted from overnight cultures of *A. baumannii* isolates using a DNA purification kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Multiplex-PCR was performed to investigate the presence of carbapenemase genes: $bla_{OXA\text{-}143}$, $bla_{OXA\text{-}23}$, $bla_{OXA\text{-}24}$, $bla_{OXA\text{-}51}$, $bla_{OXA\text{-}58}$ using primers and amplification conditions as described previously.[39] The primers used are listed in the **Table 1**.

Quantitative biofilm formation assay

Clinical XDR *A. baumannii* isolates were analyzed for their ability to produce biofilm using microtiter plate method based on the crystal violet staining method.[40] Briefly, isolates were initially cultured in the Tryptic Soy Broth (TSB) medium comprising 1% glucose. After incubation for 24 hours at 37°C, 20 μl of fresh bacteria containing medium was transferred to the 96-well polystyrene microtiter well containing 180 μl of fresh TSB medium. After incubation at 37°C for 24 hours, the medium was discarded and the adherent cells washed twice with the phosphate-buffered saline (PBS) (pH7.4) followed by addition of 250 μl (99%) methanol and then 200 μl (0.1%) Crystal Violet. The stain was eluted from the adherent cells using 160 μl acetic acid (33%). Wells containing no bacteria were used as controls. Absorbance (optical density) of the eluted solvent was measured for the clinical isolates (ODi) and negative control (ODc) at 570 nm using an UV visible spectrophotometer (Epoch, Biotek). The assay was repeated at least three

times using fresh samples each time. The results were interpreted as follows: if ODi < ODc, the bacteria were non-adherent; if ODc < ODi_2×ODc, the bacteria were weakly adherent; if 2×ODc < ODi ≤4×ODc, the bacteria were moderately adherent; and if 4×ODc < ODi, the bacteria were strongly adherent.[40]

Detection of biofilm (*bap, PER, bfmSR, csuE, pgaA*, and *pgaD*), motility (*type I fimbriae*, *PilT*), autoinducer synthase (*abaI*) and virulence related genes (*omp*)

The bacterial genomic DNA was extracted as stated above according to manufacturer's protocol. Amplification of biofilm associated, autoinducer synthase, virulence and surface motility-related genes was performed using specific primers (listed in **Table 1**) and PCR conditions in XDR *Acinetobacter baumannii* isolates.[41,42]

A. baumannii sequence-based typing method

Two sets of multiplex-PCR (SG1 and SG2) were performed with primers targeting *ompA*, *csuE* and $bla_{OXA\text{-}51\text{-}like}$ specific alleles.[30] PCR program included an initial denaturation at 94°C for 3 min followed by 30 cycles of 45 s denaturation at 94°C, 45 s primer annealing at 57°C, 1 min extension at 72°C; and a final elongation step of 72°C for 5 min.[30]

A. baumannii PCR-based replicon typing (AB-PBRT) method

The bacterial genomic DNA was extracted from overnight cultures of *A. baumannii* isolates in LB broth using a DNA purification kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. The primers used for AB-PBRT

Table 1. Primers and amplification conditions for various traits studied in the clinical *A. baumannii* isolates

Trait	*Gene*	Sequence	Size	Amplification temperature	Ref Reference
Virulence	*ompA-F*	AGCATAAAGAAGCTACACCTGC	154bp	60.5	(41)
	ompA-R	AAAGTCGCCAAGAAACCTTGAT			
Pili and motility	*fimI-F*	GACATTGGTAGCTGCACCAG	150bp	58.5	(41)
	fimI-R	GATGTTGCTGGTCGTACACC			
	PilT-F	AGTGTACCAAACACCAAGTGAC			
	PilT-R	TCGGGTAAATCAACTACGCTTG	284bp	60	
Biofilm, quorum sensing, Autoinducer synthase	*bfmR-F*	GGATCTTGTGGTCTTGGATGTC	384bp	61	(41)
	bfmR-R	GATAAAATACGGCCAGCGTTTG			
	bfmS-F	CACGTATTCGCTTTGGTACAGA	474bp	60	
	bfmS-R	GGCTATCATCTAAACGGGCAAA			
	csuE-F	TTGGCTTTAGCAAACATGACCT	564bp	58	
	csuE-R	TTGCGGGGAAAGTCCATTATTT			
	pgaA- F	GCAAATGAATCCTTCCGATCCT	670bp	57.5	
	pgaA- R	GTTTTGAGTCGTTTTTCGCCAT			
	bap-F	GGTACAAACTATGTGCCGGATT	934bp	61	
	bap-R	CTGTATTCACTCCTTGACCAGC			
	abaI-F	CCACACAACCCTATTTACTCGG	121bp	58	
	abaI-R	GGCGGTTTTGAAAAATCTACGG			
	pgaD-F	TTGATCAGCCTGAATATGTGA	145bp	60	(42)
	pgaD-R	CACACATAGTCATAAATGAGG			
Antibiotic Resistance	*blaPER-F*	ATGAATGTCATTATAAAAGC	925bp	51	(72)
	blaPER-R	AATTTGGGCTTAGGGCAGAA			
	OXA-23-F	GATCGGATTGGAGAACCAGA	501bp	52	(39)
	OXA-23-R	ATTTCTGACCGCATTTCCAT			
	OXA-40 -F	GGTTAGTTGGCCCCCTTAAA	246bp		
	OXA-40-R	AGTTGAGCGAAAAGGGGATT			
	OXA-51-F	TAATGCTTTGATCGGCCTTG	353bp		
	OXA-51-R	TGGATTGCACTTCATCTTGG			
	OXA-58-F	AAGTATTGGGGCTTGTGCTG	599bp		
	OXA-58-R	CCCCTCTGCGCTCTACATAC			
	OXA-143-F	TTCTGTCAGTGCATGCTCATC	728bp		
	OXA-143-R	CAGGCATTCCTTGCTTCATT			

are listed in **Table 2**. The PCR amplifications for 19 *rep* genes were grouped as 11 simplex PCR or as duplex PCR (**Table 2**). PCR amplifications were performed as follows: 1 cycle of denaturation at 94°C for 7 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing (mentioned in **Table 2**) and elongation at 72°C for 1.5 min. The amplification was ended with an extension program at 72°C for 5 min.

Statistical analysis

As all thirty *A. baumannii* isolates were positive for almost all the genes studied for biofilm, autoinducer synthase, virulence and surface motility-related, thus only the frequencies were calculated as percentages. The association between the genes involved in biofilm formation and the amount of biofilm mass with antibiotic resistant phenoty-

Table 2. Primers and annealing conditions for plasmid-replicon typing of *A. baumannii* isolates

Replicase groups	*Gene*	Sequence	Size	Amplification temperature	Ref
Group 1	*gr1-F*	CATAGAAATACAGCCTATAAAG	330bp	52	(27)
	gr1-R	TTCTTCTAGCTCTACCAAAAT			
Group 2	*gr2-F*	AGTAGAACAACGTTTAATTTTATTGGC	851bp	52	
	gr2-R	CCACTTTTTTTAGGTATGGGTATAG			
	gr3-F	TAATTAATGCCAGTTATAACCTTG	505bp		
	gr3-R	GTATCGAGTACACCTATTTTTTGT			
Group 3	*gr4-F*	GTCCATGCTGAGAGCTATGT	508bp	50	
	gr4-R	TACGTCCCTTTTTATGTTGC			
	gr9-F	GCAAGTTATACATTAAGCCT	191bp		
	gr9-R	AAAAATAAACGCTCTGATGC			
Group 4	*gr5-F*	AGAATGGGGAACTTTAAAGA	220bp	50	
	gr5-R	GACGCTGGGCATCTGTTAAC			
	gr11-F	GGCTATTCAAAACAAAGTTAC	852bp		
	gr11-R	GTTTCCTCTCTTACACTTTT			
Group 5	*gr6-F*	AGCAAGTACGTGGGACTAAT	662bp	50	
	gr6-R	AAGCAATGAAACAGGCTAAT			
	gr16-F	CTCGAGTTCAGGCTATTTTT	233bp		
	gr16-R	GCCATTTCGAAGATCTAAAC			
Group 6	*gr7-F*	GAACAGTTTAGTTGTGAAAG	885bp	50	
	gr7-R	TCTCTAAATTTTTCAGGCTC			
	gr18-F	TCGGGTTATCACAATAACAA	676bp		
	gr18-R	TAGAACATTGGCAATCCATA			
Group 7	*gr8-F*	AATTAATCGTAAAGGATAATGC	233bp	50	
	gr8-R	GACATAGCGATCAAATAAGC			
	gr14-F	TTAAATGGGTGCGGTAATTT	622bp		
	gr14-R	GCTTACCTTTCAAAACTTTG			
Group 8	*gr10-F*	TTTCACTAGCTACCAACTAA	371bp	50	
	gr10-R	ACACGTTGGTTTGGAGTC			
	gr13-F	CAAGATCGTGAAATTACAGA	780bp		
	gr13-R	CTGTTTATAATTTGGGTCGT			
Group 9	*gr12-F*	TCATTGGTATTCGTTTTTCAAAACC	165bp	52	
	gr12-R	ATTTCACGCTTACCTATTTGTC			
	gr15-F	GGAAATAAAAATGATGAGTCC	876bp		
	gr15-R	ATAAGTTGTTTTTGTTGTATTCG			
Group 10	*gr17-F*	AATAACACTTATAATCCTTGTA GCAAATGT-	380bp	50	
	gr17-R	GACCTCTAATATA			
	gr19-F	ACGAGATACAAACATGCTCA	815bp		
	gr19-R	AGCTAGACATTTCAGGCATT			

pes of *A. baumannii* was evaluated using software IBM SPSS Statistics version 25.0 (IBM Corp., USA). The analysis was performed with a confidence level of 95%. *P* values < 0.05 were considered statistically significant.

RESULTS

Bacterial isolates

Of 30 XDR *A. baumannii* clinical isolates enrolled in the study, 16 (53.3%) isolates were obtained from male patients and 14 (46.6%) from females. *A. baumannii* isolates were recovered from tracheal aspirate and wounds (each n=9; 30%), blood (n=7; 23.3%), IV catheter (n=1; 3.3%), and urine (n=4; 13.3%).

Antibiotic susceptibility testing

On disk diffusion assay, all isolates showed no zone of inhibition around the following disks: ceftazidime, cefepime, amikacin, gentamicin, ceftriaxone, trimethoprim-sulphamethoxazole, ciprofloxacin, ampicillin-sulbactam, imipenem and meropenem. MIC of imipenem and meropenem was >32 µg/ml. MIC of colistin ranged from 0.125 to 2 µg/ml with MIC_{50} and MIC_{90} being 0.5 and 1 respectively, thus no isolate was found resistant to colistin. MIC of tigecycline also ranged from 0.125 to 2 µg/ml with MIC_{50} and MIC_{90} being 0.5 and 2, respectively. Thus, all 30 isolates were XDR.

Detection of carbapenemase production genes by Multiplex-PCR

The carbapenem resistant *A. baumannii* (CRAB) isolates examined for the presence of five bla_{OXA} genes by PCR showed that $bla_{OXA\text{-}51}$ gene was present in all CRAB strains while, 27/30 (90%) isolates were positive for $bla_{OXA\text{-}23}$ and 46.6% strains displayed $bla_{OXA\text{-}24}$ gene. The $bla_{OXA\text{-}58}$ and $bla_{OXA\text{-}143}$ genes did not manifest in any of the CRAB strains.

Quantitative biofilm formation assays

All *A. baumannii* isolates were able to form varying degrees of biofilm. The mean optical densities for isolates ranged from 0.06 nm to 0.51 nm; however, no significant result was obtained when optical densities were compared with the presence of biofilm genes. Based on the results, biofilm formation capabilities of the isolates were classified weak, moderate, and strong biofilm producer. Of 30 XDR *A. baumannii* isolates, 19/30 (63.3%), 7/30 (23.3%), and 4/30 (13.3%) isolates displayed weak, moderate, and strong adherence activity in the microplate assay, respectively.

Detection of biofilm, motility, autoinducer synthase and virulence genes by PCR

Among 30 *A. baumannii* isolates, all were positive for biofilm-associated and autoinducer genes comprising *bfmSR, csuE, pgaA, abaI* and *pgaD*. However, detection rates of *bap* and *bla-PER1* were 76.6% (n=23) and 83.3% (n=25), respectively. However, the mean for biofilm biomass in *bap,* and *blaPER-1* positive isolates were 0.06 nm to 0.37 nm and 0.06 to 0.51 nm respectively. All isolates were also positive for t*ype I fimbriae, PilT* motility related genes, and *ompA* virulence gene.

Tri-locus sequence typing

Sequence-based typing revealed all isolates shared the same combination of alleles at the three loci belonged to only one group 1 (European clone II). No isolate belonged to Group 2 or Group 3 or any new variant.

PCR-based replicon typing (AB-PBRT) method

After analyzing the presence of *rep* genes, the results showed that *rep6* and *rep2* genes had highest frequency (75% and 65%, respectively), followed by *rep3*, *rep4*, *rep5*, *rep17* with 15%, 37%, 3%, 50% frequency, respectively. No isolate belonged to replicase groups 1, 7-16, 18, and 19. **Figs 1** and **2** depicts the genetic characterization of predominant *rep 6* and *rep 2* positive *A. baumannii* isolates. **Table 3** depicts the comparative result of Tri-locus sequence typing and PCR-based replicon typing methods utilized for typing the XDR *A. baumannii* clinical isolates.

DISCUSSION

Initially, *A. baumannii* emerged as an opportunistic pathogen and the treatment of infection was managed with β-lactams. The persistent presence of *A. baumannii* in the hospital setting allowed it to encounter antibiotics which lead to the emergence of successful clones with particular antibiotic resistance characteristics.[43] Currently, *A. baumannii* marks the culmination of organisms facing antibiotic resistance, the so-called paralyzing situation in therapeutics. The organism is notorious for causing serious infections in intensive care units (ICUs).[44] In our study, 73.3% of clinical isolates were obtained from ICUs. Unfortunately, all isolates were resistant to antibiotics commonly used by infectious specialist in our hospital setting except colistin and tigecycline. Earlier research studies displays this organism as MDR[45] while, later published research studies marked them as XDR[46] or PDR.[47] More than 90% clinical isolates were resistant to ceftazidime, cefotaxime, cefepime, amikacin, ciprofloxacin, piperacillin-tazobactam, ampicillin-sulbactam and co-tri-

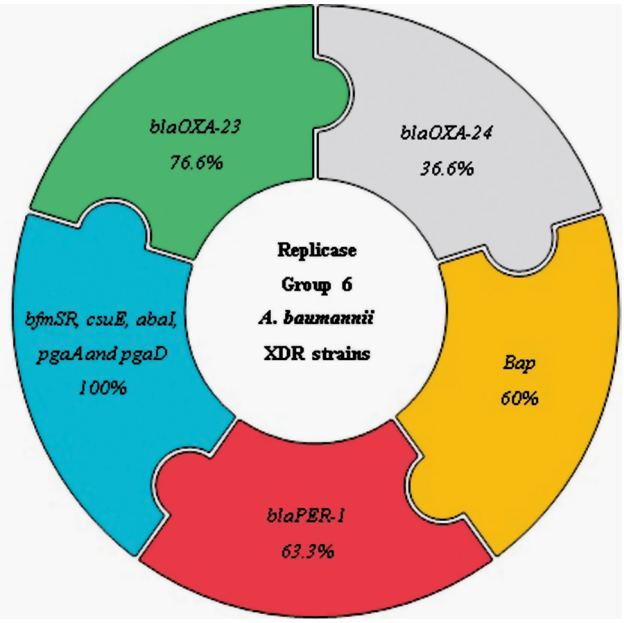

Figure 1. Genetic characterization of Group 6 replicon positive XDR *A. baumannii* strains.

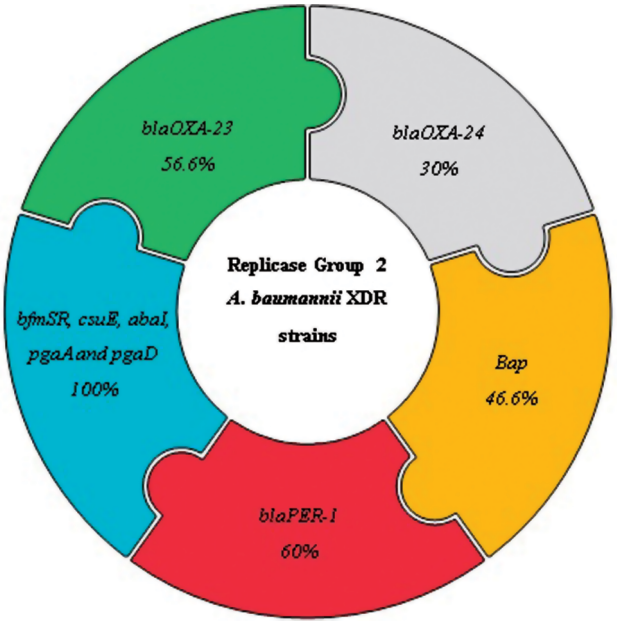

Figure 2. Genetic characterization of Group 2 replicon positive XDR *A. baumannii* strains.

moxazole in an Iranian study performed on clinical isolates obtained from two hospitals.[44]

Carbapenem was once considered as an effective drug for the treatment of infections caused by *A. baumannii*, but in recent decades, the rate of carbapenem-resistant *A. baumannii* strains has increased dramatically and is spreading throughout the world.[48-51] Studies from Asian countries including China, Thailand, and Taiwan[52-54], show carbapenem resistance to vary from 40 to 60%. In general, carbapenem-hydrolyzing class D β-lactamases (CHDLs) in *A. baumannii* is mainly mediated by bla_{OXA-23}, and $bla_{OXA-51-like}$.[55] bla_{OXA-23} are the most common OXA type of carbapenem resistance in Iran.[56] In the present investigation performed on XDR strains, all isolates had $bla_{OXA-51-like}$ gene while the frequency of bla_{OXA-23} and bla_{OXA-24} was 90% and 46.6%, respectively. We did not find presence of bla_{OXA-58} and $bla_{OXA-143}$ genes in any isolate. In most studies performed on CHDLs, bla_{OXA-58} gene has not been found[57,58] while, bla_{OXA-23} has been reported as an abundant CHDLs, with a frequency of over 80%.[12,59,60]

A study of samples collected from 1991 to 2011 showed that the content of the bla_{OXA} gene in *A. baumannii* changed from $bla_{OXA-24/40}$ and bla_{OXA-58} to bla_{OXA-23}. Since 2008, the bla_{OXA-23} gene has been the most abundant type of OXA among *Acinetobacter baumannii* species around the world. The study also showed that with the change in OXA content, the strains' ability to bind and form biofilm has decreased.[61] The results of our studies and studies in other parts of the world and in Iran show that this bacterium is resistant to carbapenem. As usage of carbapenem is the last resort treatment strategy, it is of great concern.

According to the results of the present investigation, 13.3% XDR *A. baumannii* strains had potentiality to form strong biofilm while other strains produced either moderate (23.3%) or weak (63.3%) biofilm. No significant relationship was observed between resistance and biofilm formation among *A. baumannii* species in our study. Contrary to the phenotypic findings, we could observe presence of biofilm genes in all *A. baumannii* isolates except *bap* and *blaPER* genes, which were not present in all strains. Study conducted in China[10] did not indicate a direct relationship between biofilm formation and increased antibiotic resistance, but could show a balance between the two so that weak and strong biofilm strains had the same resistance level. In addition, biofilm production was proposed as a mechanism for survival in strains that have less resistance. Another study also found no difference in biofilm formation in MDR and non-MDR strains.[61] By contrast, a strong association between biofilm formation and drug resistance has been reported in an Iranian study.[62] An Iranian study indicated high prevalence of *ompA, csuE* and *bfmSR* genes in XDR strains.[63] Compatible to another studies[62,64,65] we found the frequency of *ompA* and *csuE* genes to be much greater than *bap* gene. All *A. baumannii* strains harboured *pgaD* and *abaI* genes in contrast to another study conducted in Iran, where quiet low frequency of *pgaD* and *abaI* genes were reported (45.3% and 14%, respectively). This difference may be due to XDR strains in our study. Studies have shown that the *abaI* gene, an autoinducer synthase, is involved in the late stages of biofilm formation, and mutation of this gene disrupts the biofilm formation process.[14] Since in our research study, all *A. baumannii* strains were positive for at least one of the biofilm-associated genes thus, *abaI* may be positive in all strains. In another Iranian study, the *abaI* gene frequency was 18%. Higher frequency in our study may be due to inclusion of only XDR strains; nevertheless, we did not find any relation between biofilm

Table 3. Comparative result of Tri-locus sequence typing and replicase typing methods for XDR *A. baumannii* clinical isolates

	Tri-locus sequence typing and SGs (sequence groups)			Replicase typing and replicase GR (groups) and genes					
***A. baumannii* (AB) isolates**	**SG1**	**SG2**	**SG3**	**GR2** ***rep2***	**GR3** ***rep3***	**GR4** ***rep4***	**GR5** ***rep5***	**GR6** ***rep6***	**GR17** ***rep17***
AB 1	+	-	-	-	+	-	-	+	-
AB 2	+	-	-	+	-	-	-	+	-
AB 3	+	-	-	+	-	-	-	+	-
AB 4	+	-	-	+	-	-	-	+	+
AB 5	+	-	-	+	-	-	-	+	+
AB 6	+	-	-	-	+	-	-	+	+
AB 7	+	-	-	+	-	-	-	+	-
AB 8	+	-	-	+	-	-	-	+	-
AB 9	+	-	-	+	-	-	-	-	+
AB 10	+	-	-	-	+	-	-	+	+
AB 11	+	-	-	+	-	-	-	+	-
AB 12	+	-	-	+	-	-	-	+	+
AB 13	+	-	-	-	+	+	-	+	+
AB 14	+	-	-	+	-	-	-	+	+
AB 15	+	-	-	-	-	+	-	+	-
AB 16	+	-	-	+	-	-	-	-	-
AB 17	+	-	-	+	-	+	-	+	-
AB 18	+	-	-	-	-	+	-	-	+
AB 19	+	-	-	+	-	-	-	-	+
AB 20	+	-	-	-	-	-	-	+	-
AB 21	+	-	-	+	-	+	-	-	-
AB 22	+	-	-	+	-	+	+	+	-
AB 23	+	-	-	-	-	+	-	+	-
AB 24	+	-	-	-	-	+	-	+	+
AB 25	+	-	-	+	-	+	-	+	-
AB 26	+	-	+	-	+	-	-	+	+
AB 27	+	-	-	+	-	+	-	-	+
AB 28	+	-	-	+	-	+	-	+	-
AB 29	+	-	-	+	-	-	-	+	+
AB 30	+	-	-	-	-	-	-	-	-

production and *abaI* gene. In the present investigation, all *A. baumannii* isolates were XDR and biofilm producers, which is a clinical apprehension. The frequency of *abaI* gene was 59.8% in a Chinese study but the isolates were not specifically XDR.[66] In a study performed in Iraq, 66% non-XDR isolates revealed presence of *abaI* gene and all these isolates were biofilm producer.[67] Based on the results of this study and other investigations, we can conclude that though *abaI* gene is related to autoinducer synthesis, it plays an important role in biofilm production and is associated with antibiotic resistance.

Despite progress in the antibiotic resistance mechanisms in *A. baumannii*, still knowledge is scarce in understanding the genetic factors that have driven the recent evolution of *A. baumannii* toward multidrug resistance.[27] *A. baumannii* may develop resistance to carbapenems through plasmid-mediated acquisition of carbapenem-hydrolyzing class D β-lactamases (CHDLs). In particular the bla_{OXA-58} and bla_{OXA-23} genes, encoding the OXA-58 and OXA-23 CHDLs respectively, have been reported from *A. baumannii* isolates collected from distant parts of the world in association with plasmids.[68,69] In the present study $bla_{OXA-51\ like}$ was present in all *A. baumannii* CHDLs producing strains and bla_{OXA-23} was observed in 90% strains while, the bla_{OXA-58} was not detected in any isolate.

In the present study, XDR *A. baumannii* were investigat-

ed by plasmid replicon typing and more than 60% *A. baumannii* strains belonged to *rep6* and *rep2* types. This suggests diffusion of the carbapenem-hydrolyzing oxacillinase genes $bla_{OXA\text{-}23}$ and $bla_{OXA\text{-}51\ like}$, known to be the sources of resistance to carbapenems in *A. baumannii* strains, are related to two multiple plasmid types.

Compatible results were obtained on 3LST multiplex PCR typing, where all *A. baumannii* strains belonged to sequence group SG1, corresponding to international clone II. Studies reported from Spain and Greece shows 100% and 99.4% *A. baumannii* isolates belonged to international clone II, respectively.[30,70] The results of our research also indicate that SG1 is more common among other groups. Recent study have shown that plasmids carrying these genes may be due to the high prevalence of carbapenem resistance through CHDLs.[27] Prior to our study, *Acinetobacter baumannii* typing had not been performed using replicase typing in Iran. In a study conducted in Italy, *A. baumannii* strains belonged to groups 6 and 2, with a frequency of 96.8% and 70%, respectively. Also, the results of these studies have shown that *rep 6* may play a major role in the horizontal transmission of resistance among members of this species.[71] Because we did not perform transferability experiment, the genetic dissemination cannot be proved. However, our results showed predominance of *rep 6* and *rep 2* genes in XDR *A. baumannii* strains.

CONCLUSIONS

The results of our study shows that we are facing a jeopardous situation. Presence of biofilm associated genes except *blaPER-1* and *Bap* and other virulence genes in all clinical isolates is a feature which confirms the endemicity of *A. baumannii* and appraises the nosocomial nature of the bacteria. Our investigation showed that all *A. baumannii* strains belonged to SG1 group and two major replicase groups, which further highlights an emergence of one type of clone and its dissemination. It is thus critical to work on their transfer ability and prevention of resistant bacterial dissemination in order to avoid further restrictions on therapeutic options. In order to evaluate new specific interventions, it is essential to gather specific data on the antibiotic resistance in *A. baumannii*. As antibiotics are gradually losing their effectiveness today, other ways such as prevention of biofilm formation or using quorum-sensing quenchers should be exploited.

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Author Disclosure Statement

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Генетическая характеристика *Acinetobacter baumannii* с широкой лекарственной устойчивостью: невообразимое препятствие

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Резюме

Введение: Инфекции, вызванные *Acinetobacter baumannii*, вызывают всё большую озабоченность в области общественного здравоохранения. Способность бактерий приобретать устойчивость к ряду широко используемых антибиотиков делает их серьёзным патогеном.

Цель: Молекулярная характеристика клинических изолятов *A. baumannii* типа экстенсивной лекарственной устойчивости (ЭЛУ) с помощью полимеразной цепной реакции.

Материалы и методы: Тридцать ЭЛУ *A. baumannii* были протестированы на наличие генов, кодирующих устойчивость к карбапенемазе, объём биоплёнки, аутоиндуктивную синтазу, вирулентность и подвижность поверхности с помощью полимеразной цепной реакции (ПЦР). Затем тип изолятов определяли с помощью репликонов на основе плазмид (Rep) (PBRT) и типирования трилокусной последовательности.

Результаты: Во всех 30 штаммах XDR *A. baumannii* были идентифицированы гены, связанные с поверхностной подвижностью, аутоиндуктивной синтазой, детерминантой вирулентности, гены, связанные с биоплёнкой, за исключением PER и *bap*, с частотами 83.3% и 76.6% соответственно. Анализ генов *rep* показал наибольшую частоту генов *rep6* и *rep2* с частотой 75% и 65% соответственно. Все штаммы ЭЛУ *A. baumannii* относятся к группе SG I (European clone II).

Заключение: Наши результаты выявили необычную пластичность *A. baumannii* ЭЛУ и утверждают, что эти штаммы стали эндемичными в нашей больнице, что может вызвать опасения в ближайшем будущем.

Ключевые слова

Acinetobacter baumannii, аутоиндуктор-синтаза, биоплёнка, гидролизующие карбапенем β-лактамазы класса D, обширная лекарственная устойчивость, типирование репликазы, типирование трилокозной последовательности, вирулентность