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Plasmid borne Carbapenem-Hydrolyzing Class D β-Lactamases (CHDLs) and AdeABC efflux pump conferring carbapenem-tigecycline resistance among *Acinetobacter baumannii* isolates harboring TnAbaRs

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ABSTRACT

Here we studied the prevalence and mechanisms of simultaneous resistance to carbapenem and tigecycline and accumulation of resistance determinants reservoirs in genome of *Acinetobacter baumannii* (*A. baumannii*) clinical isolates. Susceptibility of the isolates were measured to 18 antimicrobial agents. Genetic diversity of the microbial population was determined using the International Clonal lineage typing (IC typing), multiple locus VNTR analysis (MLVA) and plasmid profiling methods. To detect the AbaRs, Carbapenem-Hydrolyzing Class D β-Lactamases (CHDLs) genes, AdeABC efflux pump genes and resistance determinants, PCR was used. Filter mating experiments were used to prove that if carbapenem resistance genes are located on conjugative plasmids or not. Among the *A. baumannii* clinical isolates, 40.8% were carbapenem-tigecycline resistant and in this population, 46.9% were belonging to IC I, IC II or IC III and 53.1% were IC variants. These isolates had fallen in 40 MLVA types and were harboring plasmids in multiple numbers and sizes. In this study, $bla_{\text{OXA-23-like}}$ was the most prevalent CHDL and conjugation analysis proved that the carbapenem resistance genes are located on conjugative plasmids. All efflux pump genes, except for *adeC*, were detected in all carbapenem-tigecycline resistant *A. baumannii* (CTRAb) isolates. Resistance determinants were distributed in both TnAbaRs and R plasmids with a shift toward the R plasmids. Emerging of carbapenem resistant *A. baumannii* (CRAB) with simultaneous resistance to the last line therapy including tigecycline represent emerging of extensively drug resistance (XDR) and pandrug resistance (PDR) phenotypes that would be a great threat to our public health system.

1. Introduction

A. baumannii has gained a massive attention in the recent decade due to its ability in developing resistance to almost all antimicrobial agents. Healthcare-associated infections (HAIs) especially in burn patients, including ventilator-associated pneumonia, bloodstream infections, wound infections, urinary tract infections and meningitis with high morbidity and mortality rate, mostly attributed to this opportunistic pathogen [1,2].

Emergence of multiple drug resistance (MDR) phenotypes among clinical isolates of *A. baumannii* is a complicated crisis worldwide including Iran and treatment of such infections would be a problematic issue [3].

Carbapenems specially imipenem and meropenem usually be used as the choice drug to treat MDR *A. baumannii* infections but there are increasing reports of CRAb infections all over the world including Iran [4,5].

Plasmid-borne genes including class B metallo-β-lactamases (MBLs) and carbapenem-hydrolyzing class D OXA-type β-lactamases (CHDLs) are the most important cause of carbapenems resistance development among *A. baumannii* clinical isolates and CHDLs; especially, are associated with the wide spread of such a resistance. Currently there are five families of CHDLs that have been

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found to be encoded by isolates of *A. baumannii*: the $bla_{OXA-23-like}$, $bla_{OXA-40-like}$, $bla_{OXA-51-like}$, $bla_{OXA-58-like}$ and $bla_{OXA-143}$ enzymes. Except for $bla_{OXA-51-like}$, which considers as an intrinsic gene, other families are acquirable and of these, the $bla_{OXA-58-like}$ and $bla_{OXA-23-like}$ genes are in association with plasmids and have been reported from *A. baumannii* isolates collected from all over the world [6–8].

Tigecycline is one of the last anti *A. baumannii* effective agents use to treat CRAb infections however tigecycline non-susceptible or resistance *A. baumannii* has been reported frequently in recent years [9,10]. To date, presence and overexpression of three RND efflux systems, AdeABC, AdeFGH, and AdeIJK, has been associated with MDR phenotypes in *A. baumannii* and among these, AdeABC is associated with reduced susceptibility to tigecycline [11].

A. baumannii clinical isolates with simultaneous resistance against carbapenems and tigecycline (CTRAb) often are resistant to almost all other anti-gram negative agents and represent emergence of XDR and PDR phenotypes which definitely is a global public health threat and demonstrate an accumulation of resistance determinants reservoirs that is a unique characterization refers to plasticity of the genome [12]. These genes reservoirs are inserted as groups of transposons in the chromosomal locus *comM* which encodes a subunit of ATPase enzyme and called *A. baumannii* resistance islands (AbaRs) and in some cases harboring more than 45 resistance genes, conferring resistance to different classes of antimicrobial agents. Also these resistance determinants could be accumulate on R plasmids [13,14].

The aim of the present study was to study the simultaneous resistance of carbapenems and tigecycline, the molecular mechanisms of such a resistance and determine the accumulation of resistance determinants on chromosomal AbaRs or R plasmids among *A. baumannii* clinical isolates.

2. Materials and methods

2.1. Bacterial isolates

A total of 120 non-repetitive *A. baumannii* isolates were collected from January 2014 to January 2015, from different specimens of hospitalized patients in Shahid Motahari hospital, a referral burn center in Tehran, Iran. Species of these isolates were initially characterized using the routine biochemical tests and then the final identification of isolates were performed by multiplex PCR using *gyrB* gene directed primers [15]. After antimicrobial susceptibility testing, 49 isolates were considered as CTRAb and these were isolated from tracheal aspiration (n = 23), blood (n = 1), CSF (n = 6), burn wound (n = 17) and urine infections (n = 2).

2.2. Antimicrobial susceptibility testing

The Clinical and Laboratory Standards Institute (CLSI) guideline (2015) [16] for Minimum Inhibitory Concentrations (MICs) using the E test was used to assess the susceptibility of 120 *A. baumannii* isolates to imipenem, meropenem, colistin and tigecycline (Ezy MIC™ strips, Himedia, India). The MIC_{50} and MIC_{90} of each antibiotic were calculated. Since there is no break point for tigecycline against *A. baumannii* strains in the CLSI guidelines; therefore, the criteria for interpretation of the MIC values of tigecycline (MIC of ≤1 mg/L defined as susceptible and >2 mg/L as resistant) were determined according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2014) for members of the *Enterobacteriaceae* sp. The CTRAb were recruited for further experiments. CLSI guideline for Bauer-Kirby disk diffusion methods (Himedia, India) was used to assess the susceptibility of CTRAb isolates to the antimicrobial agents including: amikacin (30 μg), ampicillin-sulbactam (10μg/10 μg), cefepime (30 μg), ceftazidime (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), meropenem (10 μg), imipenem (10 μg), piperacillin (100 μg), piperacillin-tazobactam (100μg/10 μg), tetracycline (30 μg), minocycline (30 μg), levofloxacin (5 μg), rifampicin (5 μg), tobramycin (10 μg), and trimethoprim-sulfamethoxazole (1.25μg/23.75 μg). *Escherichia coli* (*E. coli*) ATCC25922, *Pseudomonas aeruginosa* ATCC27853 and *E. coli* ATCC35218 were used as quality control organisms. The phenotype of *A. baumannii* was defined as MDR, XDR or PDR according to the International Expert proposal for Interim Standards Guidelines [17].

2.3. Phenotypic detection of MBLs

Metallo β-lactamases (MBLs) detection was done by imipenem-EDTA combined disk method [18].

2.4. Conjugation experiments

To determine if carbapenem resistance genes are located on conjugative plasmids or not, filter mating experiment was performed using the *A. baumannii* isolates; TEH254, resistant to imipenem and susceptible to trimethoprim-sulfamethoxazole, and TEH123, susceptible to imipenem while resistant to trimethoprim-sulfamethoxazole, as donor and recipient cells, respectively. Equal amounts of overnight cultures of the donor and recipient cells were mixed and incubated on a Brain Heart Infusion agar (BHI) plate overnight. Transconjugants were selected on BHI plates containing imipenem (32 mg/L) plus trimethoprim-sulfamethoxazole (250 mg/L). The frequency of transfer was calculated as the number of transconjugants divided by the number of surviving recipients [19].

2.5. DNA extraction and plasmid profiling

Total DNA and plasmid DNA used as template for PCR amplifications were extracted using the High Pure PCR Template Preparation Kit and High Pure Plasmid Isolation Kit respectively (Roche Inc., Mannheim, Germany) following the manufacturer's instruction. In purpose of plasmid profiling, plasmid DNA was separated by electrophoresis on 0.5% (w/v) agarose gels, stained with ethidium bromide (5 mg/L), visualized with UV and imaged using a Gel Doc TM XR image analysis station (Bio-Rad, Hercules, USA). Product sizes were estimated using the 10 kb DNA ladders (New England BioLabs, USA) as molecular size markers.

2.6. Identification of international clone types (IC) and multiple locus VNTR analysis (MLVA)

IC types were determined based on presence or absence of alleles of the outer membrane protein A (*omp*A), chaperone–subunit usher E (*csu*E), and the intrinsic carbapenemase ($bla_{OXA-51-like}$) encoding genes in the two simultaneously multiplex PCR as previously described [20]. All isolates were grouped in three major ICs (ICI, ICII or ICIII) and several so-called PCR-based groups (SGs).

Also the isolates were genotyped using the MLVA-8 scheme method previously described [21]. For clustering analysis, the allele strings were entered into the BioNumerics software v.7.0 (Applied maths, Sint-Martens-Latem, Belgium) as character values. A cut-off value of 80% similarity was applied to define clusters.

2.7. Molecular screenings

AbaRs detection was performed in a two steps strategy including (1) PCR determination of ATPase presence and (2) PCR determination of interruption of the ATPase gene using the primers and methods previously described [14].

PCR was used to detect the β-lactamases genes includes: class A (*bla*$_{TEM}$, *bla*$_{VEB}$), class B or MBLs (*bla*$_{IMP-1}$, *bla*$_{VIM-2}$, *bla*$_{GIM-1}$, *bla*$_{SPM-1}$ *bla*$_{SIM-1}$, *bla*$_{NDM-1}$), class C or cepholsoporinase (*bla*$_{ADC}$) and class D (*bla*$_{OXA-23-like}$, *bla*$_{OXA-40-like}$, *bla*$_{OXA-51-like}$, *bla*$_{OXA-58-like}$ and *bla*$_{OXA-143}$) among *A. baumannii* isolates using the primers described previously [22–26].

To determine whether IS*Aba1* was present upstream of *bla*$_{OXA-23-like}$, *bla*$_{OXA-51-like}$ and *bla*$_{ADC}$ genes, PCR mapping experiments were performed [27,28]. Efflux system genes; *adeA*, *adeB*, *adeC*, *adeS* and *adeR* were detected using the primers described previously [29].

Twenty resistance genes, conferring resistance to different classes of antimicrobial agents including β-lactamases, aminoglycosides, tetracycline, chloramphenicol, sulphonamides, trimethoprim, rifampicin, mercuric, arsenic and cadmium ions were assessed by PCR using the primers previously described [14] tested with both total DNA and plasmid DNA to determine whether these genes located on AbaRs or plasmids. Detection was first performed with total DNA and then with plasmid DNA. The results of detection with plasmid DNA considered as plasmidal (R plasmids) location and subtraction of total DNA results from plasmid DNA results considered as chromosomal (AbaRs) location.

2.8. Statistics analysis

Statistical analysis was performed using the "IBM SPSS statistics 22" software (IBM analytics; USA). Statistical significance of variables determined by chi-square and Fisher's exact tests. Level for statistical significance of variables considered as P value ≤ 0.05.

3. Results

3.1. A high level of simultaneous resistance against carbapenems and tigecycline was observed

In this study carbapenem resistance rate was 75.8% (91 from 120 isolate) and from these, 53.8% (49 from 91) had simultaneous resistance to tigecycline. By MIC screening for detection of decreased susceptibility to tigecycline among 120 *A. baumannii* isolates, 40.83% of the isolates (n = 49) were confirmed to be isolates with decreased susceptibility to tigecycline. Among these CTRAb isolates, high susceptibility were observed to colistin (100%) with MIC_{50} values of 0.38 mg/L and MIC_{90} values of 0.5 mg/L (Table 1) and from these, 12.2% (n = 6) showed MDR phenotypes and 87.8% (n = 43) showed XDR phenotypes. After colistin and polymyxin (100% susceptibility) the most effective antimicrobial agent among CTRAb isolates was the ampicillin-sulbactam with 26.5% susceptibility. The resistance rate of CTRAb isolates to imipenem and meropenem were 98% and 100% respectively. The antibiotic susceptibility pattern of CTRAb isolates to other antimicrobial agents are reported in Table 2.

None of the CTRAb isolates showed MBLs production in imipenem-EDTA combined disk method. The molecular detection for *bla*$_{MBL}$ genes including *bla*$_{IMP-1}$, *bla*$_{VIM-2}$, *bla*$_{GIM-1}$, *bla*$_{SPM-1}$ *bla*$_{SIM-1}$ and *bla*$_{NDM}$, were negative too. The intrinsic carbapenem and cephalosporins resistance genes includes *bla*$_{OXA-51-like}$ and *bla*$_{ADC}$ were detected in all CTRAb isolates. The IS*Aba1* was detected upstream of 87.7% of the *bla*$_{OXA-51}$ and 10.2% of the *bla*$_{ADC}$. The prevalence of CHDLs including *bla*$_{OXA-23}$, *bla*$_{OXA-40}$, *bla*$_{OXA-58}$ and *bla*$_{OXA-143}$ were 83.7 (n = 41), 12.2% (n = 6), 0% and 0% respectively. IS*Aba1* was detected upstream of all *bla*$_{OXA-23-like}$ positive isolates and there was a significant association between the presence of IS*Aba1* and imipenem MIC (MIC $\geq$ 32 mg/L) (P value = 0.000) (see Table 3).

Table 1
The minimum inhibitory concentration (MIC) range, MIC_{50} and MIC_{90} distribution of 4 antimicrobial agents for 49 CTRAb isolates as determined by E test.

Antimicrobial agent	Range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)
Tigecycline	2–16	2	3
Colistin	0.01–0.5	0.38	0.5
Meropenem	1.5–32	32	32
Imipenem	3–32	32	32

3.2. The carbapenem resistance genes were located on conjugative plasmids

To study the mechanism responsible for imipenem resistance, we tested the possibility of imipenem resistance transfer through conjugation. Filter-mating experiments demonstrated that resistance to imipenem was transferred from isolate TEH254 to imipenem-susceptible isolate TEH123 at a frequency ranging from 1.5×10^5 to 1.5×10^6. Antimicrobial susceptibility profile of the transconjugants was identical to that of the recipient isolate TEH123 with except for imipenem and meropenem. Imipenem MICs for transconjugants were similar (32 m/L) to those for donor isolates and were inhibited in the presence of 200 mM NaCl, showing that in the transconjugants, imipenem resistance was induced by oxacillinase activity.

3.3. The CTRAb isolates showed a high level of genetic diversity

The population structure of CTRAb isolates was very diverse and belonging to multiple clones. The IC I (G2), IC II (G1) and IC III (G3) prevalence were 40.8, 4.1 and 2% respectively and 53.1% were new IC variants or SGs. Among these SGs, G9 was the most prevalent SG variant. The prevalence of other SGs are showed on Table 4. By the MLVA typing method, the 49 CTRAb isolates were grouped into 40 distinct MLVA types with 4 clusters and 33 singleton genotypes (Fig. 1). All MLVA- Loci were present in all studied isolates. In this study, the VNTR loci MLVA-AB_3530, MLVA-AB_3002 and MLVA-AB_2240 displayed lower diversity, whereas VNTR loci MLVA-AB_2396 and MLVA-AB_0845 showed higher level of diversity. All CTRAb isolates were carrying plasmids with diversity in numbers and sizes. Each isolates was harboring 2–5 plasmids with size ranging from 2 kb up to 10 kb and there were common in a 10 kb plasmid.

3.4. The CTRAb isolates all had AbaRs and AdeABC efflux system

ATPase gene were present in all isolates and the interruption has occurred in all of these, indicating that all these isolates harboring the AbaRs. Efflux system genes *adeA* and *adeB*, were present in all isolates but *adeC* were observed in only 20.4% of the isolates. The *adeS* and *adeR* genes were present in all CTRAb isolates too.

3.5. The resistance genes reservoirs distributed on both TnAbaRs and R plasmids

From over 20 resistance genes tested in this study, *bla*$_{TEM}$ was detected in all CTRAb isolates. Among these, 67.3% were located on the chromosomal TnAbaRs and 32.7% on R plasmids. In this study *bla*$_{VEB}$ was not observed. The rifampicin resistance gene *arr-2* was detected in all CTRAb isolates too but all were located on TnAbaRs and none on plasmids. The aminoglycoside encoding gene *strB* were positive in all isolates and from these, 61.8% were located on TnAbaRs while 38.8% were located on R plasmids (Fig. 2). The results for detection of resistance genes reservoirs on TnAbaRs and R plasmid are showed in Table 5 and Fig. 2.

Table 2
Frequency of antimicrobial resistance to CLSI antimicrobial groups in three major epidemic lineages among 49 CTRAb isolates. IC, international clonal lineage; V, IC variants; AMK, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; CST, colistin; FEP, cefepime; GEN, gentamicin; IPM, imipenem; LVX, levofloxacin; MEM, meropenem; MIN, minocycline; PIP, piperacillin; RIF, rifampicin; SAM, ampicillin-sulbactam; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline; TGC, tigecycline; TOB, tobramicin; TZP, piperacillin-tazobactam. A: Agents in Group A are considered appropriate for inclusion in a routine, primary testing panel, as well as for routine reporting of results for the specific organism groups. B: Group B includes antimicrobial agents that may warrant primary testing but they may be reported only selectively, such as when the organism is resistant to agents of the same class, as in Group A. O: Group O ("Other") Includes antimicrobial agents that have a clinical indication for the organism group, but are generally not candidates for routine testing and reporting in the United States.

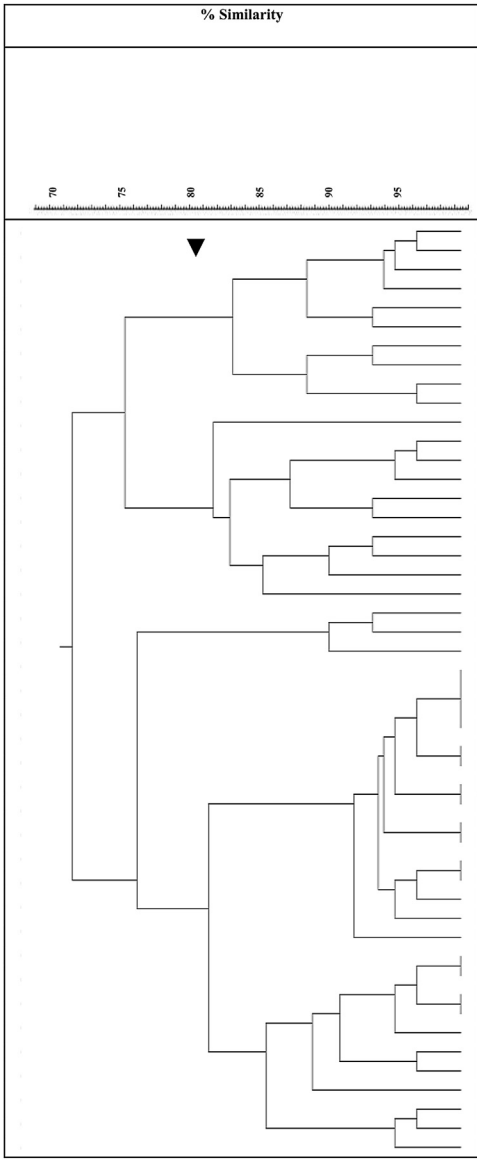
% resistance to CLSI antimicrobial groups																		
IC (No)	A								B								O	
	IPM	MEM	CAZ	SAM	AMK	TOB	GEN	CIP	PIP	TZP	FEP	MIN	TET	TGC	LVX	SXT	CST	RIF
IC I (20)	95	100	100	80	95	80	95	95	100	100	100	90	95	95	95	80	0	100
IC II (2)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	0	100
V (27)	100	100	100	66.7	100	100	100	100	100	100	100	70.4	92.6	92.6	92.6	100	0	100
Total (49)	98	100	100	73.5	98	91.8	98	98	100	100	100	79.6	93.9	93.9	93.9	91.8	0	100

Table 3
Isolates characteristics, OXA types, ISAba1 Upstream mapping of oxacillinase genes, AdeABC efflux system, and AbaR detection among 49 CTRAb isolates.

Isolates characteristics			OXA TYPE					ISAba1 Upstream mapping of oxacillinase genes			AdeABC efflux system					AbaR Detection	
ID	WARD	SPECIMEN	OXA-51	OXA-23	OXA-41	OXA-58	OXA-143	$bla_{OXA-23-like}$	$bla_{OXA-51-like}$	bla_{ADC}	adeA	*adeB*	*adeC*	*adeR*	*adeS*	*ATPase presentation*	*ATPase interruption*
16	ICU	CSF	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+
123	ICU	CSF	+	+	−	−	−	+	+	−	+	+	+	+	+	+	+
124	ICU	CSF	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+
125	ICU	CSF	+	+	−	−	−	+	−	−	+	+	+	+	+	+	+
156	ICU	wound	+	+	−	−	−	+	+	+	+	+	−	+	+	+	+
175	ICU	tracheal	+	+	−	−	−	+	+	−	+	+	+	+	+	+	+
177	ICU	tracheal	+	−	−	−	−	+	+	−	+	+	−	+	+	+	+
181	ICU	wound	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+
183	ICU	tracheal	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+
185	ICU	tracheal	+	−	−	−	−	+	+	−	+	+	+	+	+	+	+
188	ICU	tracheal	+	+	−	−	−	+	−	−	+	+	−	+	+	+	+
189	ICU	wound	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+
239	ICU	tracheal	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+
254	ICU	tracheal	+	+	−	−	−	+	+	+	+	+	−	+	+	+	+
255	ICU	wound	+	+	−	−	−	+	+	−	+	+	+	+	+	+	+
261	ICU	wound	+	+	−	−	−	+	−	−	+	+	−	+	+	+	+
264	ICU	wound	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+
265	ICU	tracheal	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+
272	ICU	tracheal	+	+	−	−	−	+	+	−	+	+	+	+	+	+	+
274	ICU	tracheal	+	+	−	−	−	+	+	+	+	+	−	+	+	+	+
294	ICU	CSF	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+
307	ICU	CSF	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+
309	ICU	wound	+	+	−	−	−	+	+	−	+	+	+	+	+	+	+
318	ICU	urine	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+
322	ICU	tracheal	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+
326	ICU	wound	+	−	+	−	−	+	+	−	+	+	−	+	+	+	+
327	ICU	wound	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+
332	ICU	urine	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+
336	ICU	wound	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+
338	ICU	wound	+	−	+	−	−	+	+	+	+	+	+	+	+	+	+
346	ICU	wound	+	+	−	−	−	+	−	−	+	+	−	+	+	+	+
348	ICU	wound	+	−	+	−	−	+	−	−	+	+	−	+	+	+	+
349	ICU	wound	+	−	+	−	−	+	+	−	+	+	−	+	+	+	+
351	ICU	wound	+	−	+	−	−	+	+	−	+	+	−	+	+	+	+
360	ICU	wound	+	+	−	−	−	+	+	+	+	+	−	+	+	+	+
379	ICU	tracheal	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+
380	ICU	tracheal	+	+	−	−	−	+	+	−	+	+	+	+	+	+	+
381	ICU	tracheal	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+
383	ICU	tracheal	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+
385	ICU	blood	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+
386	ICU	tracheal	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+
387	ICU	tracheal	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+
389	ICU	wound	+	−	+	−	−	+	+	−	+	+	+	+	+	+	+
394	ICU	tracheal	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+
399	ICU	tracheal	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+
413	ICU	tracheal	+	+	−	−	−	+	−	−	+	+	−	+	+	+	+
414	ICU	tracheal	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+
428	ICU	tracheal	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+
430	ICU	tracheal	+	+	−	−	−	+	+	−	+	+	−	+	+	+	+

Table 4
Combinations of amplicons obtained in the dual multiplex PCRs used to describe 49 CTRAb international clonal (IC) lineages.

Variant type (No.)	PCR group 1			PCR group 2		
	csuE 702bp	$bla_{OXA\text{-}51\text{-}like}$ 559bp	*ompA* 355bp	*csuE* 580bp	*ompA* 343bp	$bla_{OXA\text{-}51\text{-}like}$ 162bp
G1 (2)	+	+	+	−	−	−
G2 (20)	−	−	−	+	+	+
G3 (1)	+	+	−	−	+	−
G6 (3)	−	−	−	+	−	−
G7 (4)	−	−	+	+	−	−
G9 (10)	−	−	+	+	−	+
G15 (1)	+	−	−	−	−	+
G16 (4)	−	+	−	+	−	−
G17 (4)	+	−	+	−	−	+



MLVA allelic profile								Strain characterizations			Antibiotics MICs(μg/ml)			
MLVA-AB_3530	MLVA-AB_3002	MLVA-AB_2240	MLVA-AB_1988	MLVA-AB_0826	MLVA-AB_0845	MLVA-AB_2396	MLVA-AB_3468	Strain ID	Resistance phenotype	G	IMIPENEM	MEROPENEM	TIGECYCLINE	COLISTIN
7	7	3	9	17	16	19	14	177	XDR	G9	32	32	2	0.38
7	7	3	9	17	19	19	14	309	MDR	G2	32	12	2	0.25
7	7	3	9	19	19	19	14	385	XDR	G2	32	32	2	0.50
7	7	4	9	19	19	19	14	414	XDR	G2	32	32	2	0.38
7	7	4	9	17	19	16	14	386	XDR	G1	32	32	3	0.50
7	8	4	9	17	19	19	14	399	XDR	G15	32	32	3	0.50
7	7	4	9	13	19	21	13	156	XDR	G9	32	32	2	0.38
7	7	4	9	17	16	21	13	383	XDR	G2	32	32	2	0.50
6	7	4	9	17	19	21	13	379	XDR	G2	32	32	2	0.25
6	7	4	9	17	19	19	13	389	XDR	G9	32	32	16	0.19
7	8	3	5.5	17	16	21	13	380	XDR	G1	32	16	2	0.25
7	7	4	9	17	16	9	13	16	XDR	G2	32	32	3	0.30
7	7	4	9	17	16	9	14	188	XDR	G17	32	32	3	0.38
7	7	4	5	17	16	9	14	332	XDR	G17	32	32	2	0.25
7	7	3	9	17	29	9	13	183	XDR	G15	32	32	2	0.50
7	7	3	5	17	16	9	13	318	XDR	G17	32	32	2	0.38
6	8	3	5.5	17	19	16	13	189	XDR	G16	32	32	3	0.38
6	7	3	5.5	17	19	9	13	272	MDR	G2	32	32	2	0.38
6	7	3	9	17	19	9	14	336	XDR	G9	32	32	2	0.25
6	8	3	9	17	16	9	13	326	MDR	G7	32	12	2	0.19
6	7	3	5.5	13	19	19	13	261	XDR	G6	32	32	2	0.25
6	8	3	5.5	13	16	19	13	294	XDR	G2	32	32	2	0.38
6	8	3	5.5	19	42	19	13	346	XDR	G2	32	32	16	0.06
7	7	4	9	17	19	19	14	307	XDR	G7	32	32	2	0.25
7	7	4	9	17	19	19	14	413	XDR	G16	32	32	2	0.38
7	7	4	9	17	19	19	14	428	XDR	G2	32	32	2	0.38
7	7	4	9	17	19	19	14	430	XDR	G2	32	32	2	0.38
7	7	4	9	17	16	19	14	175	XDR	G9	32	32	2	0.38
7	7	4	9	17	16	19	14	185	XDR	G2	32	32	3	0.38
7	7	4	5	17	16	19	14	181	XDR	G9	32	32	3	0.38
7	7	4	5	17	16	19	14	338	MDR	G2	32	32	2	0.25
7	7	4	5.5	17	16	19	14	351	XDR	G9	32	32	3	0.12
7	7	4	5.5	17	16	19	14	387	XDR	G17	32	32	2	0.38
6	7	4	9	17	19	19	14	322	XDR	G16	32	32	2	0.38
6	7	4	9	17	19	19	14	327	XDR	G2	32	32	3	0.38
6	7	4	9	17	16	19	14	348	XDR	G3	32	32	16	0.01
6	7	4	5.5	17	16	19	14	349	XDR	G2	32	32	3	0.12
6	7	3	5.5	17	16	19	14	394	XDR	G2	32	32	2	0.38
6	7	3	5.5	19	19	9	14	123	XDR	G2	3	1.5	2	0.38
6	7	3	5.5	19	19	9	14	124	XDR	G2	32	16	2	0.25
6	7	3	5.5	17	19	9	14	274	XDR	G7	32	32	2	0.19
6	7	3	5.5	17	19	9	14	360	MDR	G15	32	32	3	0.25
6	7	3	5.5	17	29	9	14	125	XDR	G9	32	32	3	0.38
6	7	3	5.5	13	42	9	14	255	XDR	G9	32	32	2	0.25
6	7	3	5.5	13	19	9	14	265	XDR	G9	32	32	2	0.19
6	7	3	5.5	13	29	19	14	264	XDR	G7	32	32	2	0.25
6	7	3	5.5	13	19	16	14	239	XDR	G2	32	24	3	0.25
6	7	3	5.5	13	16	16	14	254	MDR	G2	32	32	2	0.38
6	7	3	5	13	16	16	14	381	XDR	G16	32	32	4	0.25

Fig. 1. Dendrogram shows the genetic diversity of 49 CTRAb isolates by MLVA; MIC ranges of colistin, imipenem, meropenem and tigecycline; resistance phenotype; and international clonal lineage.

4. Discussion

CRAb isolates simultaneously resistant to non-β-lactam antimicrobial agents are considering as a global threat to public health systems according to therapeutic options limitation. Tigecycline and colistin are often the only treatment options for CRAb infections but resistance to both agents has recently been described [30–32].

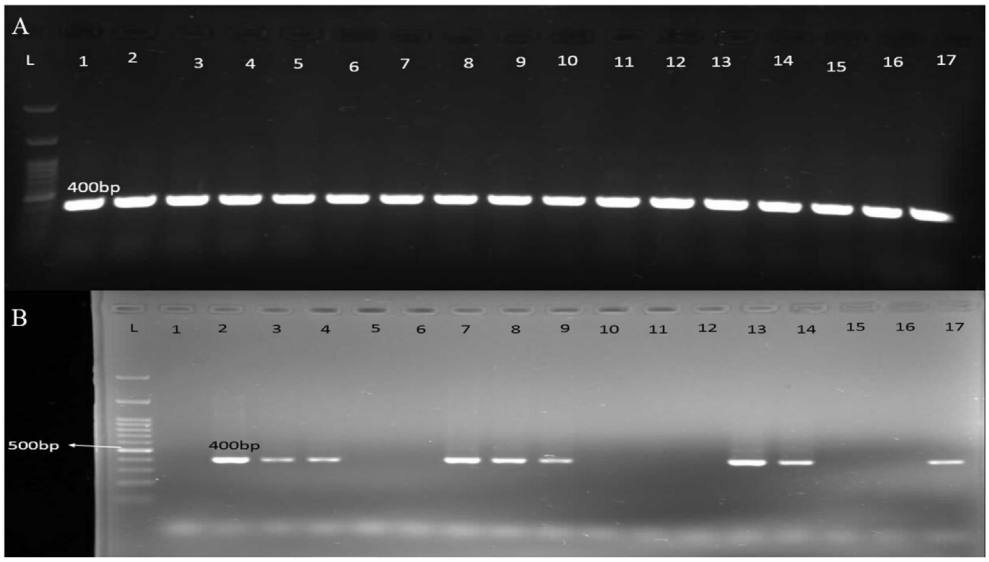


Fig. 2. Gel electrophoresis for *strB* gene (400bp) encoding for aminoglycoside resistance. A: The CTRAb isolates tested for *strB* gene with total DNA, B: The same isolates tested with plasmid DNA. L: 100bp DNA marker. 1–17: clinical isolates of *A. baumannii.*

Table 5
Distribution of resistance determinants on TnAbaRs and R plasmids among 49 CTRAb isolates.

Gene	Conferring resistance to	AbaR (%)	Plasmid R (%)	Total (%)
bla_{TEM-1}	Betalactames	67.3	32.7	100
bla_{VEB-1}		0	0	0
aac(3)-Ia	Aminoglycoside	79.6	20.4	100
aph(3′)-Ia		67.3	18.4	85.7
ant(3″)-Ia		85.7	10.2	95.9
ant(2″)-Ia		22.4	77.6	100
strA		59.2	34.7	93.9
strB		61.2	38.8	100
tet-A	Tetracycline	14.3	44.9	59.2
tet-R		100	0	100
cmlA1	Chloramphenicol	49	16.3	65.3
catA1		100	0	100
sul1	Sulphonamide	95.9	4.1	100
dfrA1	Trimethoprim	16.3	2.1	18.4
merA	Mercuric ion	98	0	98
merP		0	0	0
merR		0	0	0
asrB	Arsenic ion	0	0	0
cadA	Cadmium ion	100	0	100
aar-2	Rifampicin	100	0	100

Simultaneous resistance of CRAb to last line therapy includes tigecycline and colistin leads to a rapid dissemination of the organism due to the lack of any efficacious drug and the clonal nature of the infections outbreaks [33]. In developing countries includes Iran, control of CRAb infections is a real problem especially in hospitalized burn patients [34,35]. Carbapenem resistance among *A. baumannii* clinical isolates in Iran was ranging between 27-32% in 2007 and 82–95% in 2015, showing an ascending pattern [36–38]. In this study carbapenem resistance was 75.8% (91 from 120 isolate). From these, 53.8% (49 from 91 isolates) had simultaneous resistance to tigecycline. Tigecycline resistance in Iran is ranging from 3 to 20% [35,39] and the tigecycline resistance prevalence in this study was 40.8%. This is a “red alert” for our public health system and should be considers as an emergence issue. In other countries tigecycline resistance rate is very diverse and range from 14 to 60%. However the restriction policy on the empirical prescription of tigecycline could leads to decrease the resistance rates [5,40,and41]. Simultaneous resistance to carbapenem and tigecycline could be refers to the monotherapy with these antibiotics and combination of effective antibiotics should be prescribed to avoid such resistances.

Among the CTRAb clinical isolates the most effective antimicrobial agents were the colistin and polymyxin which none resistance was observed to them. This may refers to not using these agents for decades because of their nephrotoxicity, but with emerging of MDR gram negative bacteria they are now the last resort antibiotics for such infections [42].

The CTRAb isolates in this study showed a large genetic diversity, in both IC typing and MLVA typing. In IC typing, the most prevalent clone was the IC I, however majority of the population were belonging to new IC variants or SGs. Due to the fact that the most prevalent clone in the world is IC II which are more susceptible to the antimicrobials, we are facing an outburst of new clones that are more persistent and showing higher levels of resistance to multiple antimicrobials. These data are consistent with the recent study performed in Iran in the same hospital [5]. MLVA typing confirmed the IC typing data, and revealed that the CTRAb isolates involved in this study, constitute a diverse population, however there was not any statistically significant correlations between the data from these two typing methods. We also confirmed the previous studies that in comparison to other molecular typing methods, MLVA typing has a higher discriminatory power to differentiate the genetically close related organisms [5,21].

Plasmid profiling has been used to study the epidemiology of *A. baumannii* previously and many reports has proved that most of *A. baumannii* isolates harboring multiple plasmids with variable molecular sizes which have roles in antibiotic resistance and the epidemiological characteristics of *A. baumannii*. Here in the present study all CTRAb were harboring multiple plasmids in deferent sizes however there was no significant correlations between the plasmid profile patterns and the antimicrobial susceptibility patterns of the CTRAb isolates [9,43].

MBLs still are rare in *A. baumannii* clinical isolates and our results confirmed the previous studies both phenotypically and molecularly [44,45]. MBLs of IMP, VIM and SIM types are often described as gene cassettes of class 1 integrons which are the most prevalent in *A. baumannii*, but due to the lack of *int1* gene which

represent the class 1 integrons in our *A. baumannii* clinical isolates, none gene cassettes or gene clusters were observed [3].

In this study presence of IS*Aba1* upstream of intrinsic carbapenem resistance gene includes $bla_{\text{OXA-51-like}}$ and the cepholsoporinase bla_{ADC} was associated with increased MICs against carbapenems and cephalosporins due to its effect in overexpression of these genes and were concordant with other studies [46,47].

We showed that the $bla_{\text{OXA-23-like}}$ was the most prevalent CHDL in this study followed by $bla_{\text{OXA-40-like}}$ and this data confirms some recent studies from our geographic area [48,49]. The mostly disseminated CHDL in the world is $bla_{\text{OXA-23-like}}$ and *A. baumannii* clinical isolates which producing these enzymes are mostly reported to be associate with hospital outbreaks [6,50and51]. In mating experiments it was revealed that imipenem resistance could be transfer from a resistant *A. baumannii* clinical isolate to a susceptible isolate that indicates the presence of carbapenems resistance genes on conjugative plasmids and recent studies confirm this data [52,53].

RND-type efflux pump AdeABC, reported to be associated with decreased susceptibility to tigecycline [54–56]. Many studies has reported the harboring of AdeABC efflux system in clinical isolates of *A. baumannii*. Some studies suggested that these genes are only present in MDR isolates and some reported them in both MDR and non-MDR isolates [57,58]. We reported the presence of *adeA*, *adeB*, *adeS* and *adeR* in all CTRAb isolates but *adeC* was less prevalent. This is comply with other studies, suggested that *adeC* is not essential for the assembly of the efflux system and the AdeAB could be assembled with other outer membrane proteins [59]. In concordant of some studies, and in addition to CTRAb isolates, we observed *adeA* and *adeB* genes in non-CTRAb isolates too, and this indicate that the efflux pumps are intrinsic for *A. baumannii* and the overexpression of these pumps in responding to expose with antibiotics leads to decrease susceptibility to tigecycline [60,61].

The success of *A. baumannii* in rapid developing resistance to new antibiotics is due to the plasticity of its genome to acquire and lose of mobile genetic elements such as plasmids and transposons that change the genomic structure of the organism [3]. Some studies suggested that the high plasticity of *A. baumannii* genome is refers to presence of IS*Aba1*, which was present in all CTRAb isolates in this study [62]. We reported interruption of ATPase gene in all CTRAb isolates indicating the presence of AbaRs in our *A. baumannii* clinical isolates. However according to many studies the rate of ATPase interruption (presence of AbaRs), the genetic structure and the gene containing of AbaRs are very diverse and sometimes contradictory [14,63].

In this study there was a balance in distribution of resistance genes reservoirs in TnAbaRs and R plasmids however a shift was observed toward the R plasmids. This may refers to the environmental pressures on the bacterial genomes such as application of antimicrobial agents that leads to gene exchange between the TnAbaRs and the R plasmids in purpose of horizontal gene transfer among the microbial population [64].

In conclusion, in this study, plasmid borne CHDLs were conferring resistance to carbapenems and insertion of IS*Aba1* was leaded to increasing MICs against these agents. On the other hand presence of AdeABC efflux pump were associated with decreased susceptibility to tigecycline however measuring the efflux pump genes expression is necessary. Resistance genes reservoirs were accumulated in both TnAbaRs and R plasmids.

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Conflict of interest

The authors have no financial conflicts of interest.

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