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Clonal Lineages and Virulence Factors among *Acinetobacter baumannii* Isolated from Southwest of Iran

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Multidrug-resistant strains of *Acinetobacter baumannii* have emerged as formidable nosocomial pathogens, particularly among patients with pneumonia in intensive care units (ICUs) with a high mortality rate. In this study 100 *A. baumannii* isolates from patients in tertiary ICUs of Shiraz university hospital in southwest of Iran were selected and tested for susceptibility to 24 antimicrobials and multiplex PCR methods were used to determine virulence factors and International Clone (IC) of *A. baumannii* isolates. Considerable number of the isolates (68%) belonged to IC2, which was widespread in all ICUs. Twenty-one (21%) isolates pertained to IC1. Eleven of the isolates (11%) belonged to 3 novel variant of SG4-6. Drug resistance pattern showed that MDR and XDR phenotype proportion among isolates was 75% and 18%, respectively. 13% of the *A. baumannii* isolates were resistant to colistin. All of the isolates were positive for both the *pld* and the *pga*B virulence genes tested. The prevalence of *ptk* and *eps*A genes was 71% and 38%, respectively. Coexistence of *ptk* with *eps*A genes was observed in 29% of the isolates. The prevalence of the *ptk* and the *eps*A genes among of isolates differs noticeably in different clonal types.

Keywords: *Acinetobacter baumannii*, International clone, Multidrug-resistance, Virulence genes.

Acinetobacter baumannii has become an important pathogen that causes several diseases, ranging from minor infections of the wound to ventilator-associated pneumonia, catheter-related blood stream infection, bacteremia and meningitis[1]. The epidemiological features of *A. baumannii*, especially multidrug resistance (MDR) strains, are varying quickly[2]. From the first MDR *A. baumannii* strain reported until the present, various healthcare-associated MDR *A. baumannii* clones have been disseminated worldwide[3,4]. The emergence of new strains of community associated MDR *A. baumannii* in many parts of the world is well documented[1,5].

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Nevertheless, several studies have shown epidemiology and resistance profiles of *A. baumannii* isolates from Iranian patients[5-8]. To date, no studies are available on the molecular epidemiology of virulence factors in *A. baumannii* isolates in Iran. Several *A. baumannii* components are thought to be associated with its pathogenicity, including the protein tyrosine kinase (PTK)[9], polysaccharide export outer membrane protein (EpsA)[9], polysaccharide deacetylase (PgaB)[10] and phospholipase D (PLD)[11]. PTK and EpsA are essential for a capsule-positive phenotype of *A. baumannii*[9]. PgaB is necessary for poly-β-(1-6)-*N*-acetylglucosamine (PNAG) export, which has been well described as a main constituent of biofilms[10]. *A. baumannii* PLD is associated with ability to succeed in serum and epithelial cell invasion[11]. Furthermore, the distribution of genes

coding virulence factors PTK, EpsA and PgaB in clinical isolates of MDR *A. baumannii* has never been reported in worldwide.

Several studies have demonstrated the geographically distribution occurrence of CRAB, which proposed a clonal relatedness of these strains. Three international *A. baumannii* clones (IC), the so-called European clones I, II and III, are implicated in outbreaks worldwide and associated with carbapenem resistant[12].Therefore, the aims of the present investigations were (i) to determine the clonal lineage of Iranian *A. baumannii* isolates, (ii) to demonestrate the potential associations between genes encoding virulence factors, either alone or together, with clonal lineage, (iii) to examine any association between the resistance profiles with clonal lineage

MATERIALS AND METHODS

Definition

A. baumannii is defined as multidrug-resistant (MDR) when the organism is resistant to at least one agent in three or more antimicrobial categories that would otherwise serve as treatments for *Acinetobacter* infection. Extensively drug-resistant (XDR) is defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. *A. baumannii* isolates remain susceptible to only one or two categories) and pandrug-resistant (PDR) is defined as non-susceptibility to all agents in all antimicrobial categories[13].

Isolates

A total of 100 non repetitive isolates of MDR *A. baumannii* were isolated from several clinical sources including the respiratory tract (n=51), wound (n=27), urine (n=12), blood (n=7), and CSF (n=3) from intensive care unit (ICU) of Nemazee Hospital, Shiraz, Iran from October 2011 to September 2012. Species of the isolates were identified initially using the API20NE system (bioMérieux, Marcy-l'Etoile, France). Detection of bla_{OXA-51} gene by polymerase chain reaction (PCR) was used to confirm *A. baumannii*[14]. They were kept at -20°C in CRYOBANK™ (Copan Diagnostics Inc., Canada) until further testing.

Antimicrobial Susceptibility Testing

The Clinical and Laboratory Standards Institute (CLSI)[15] guideline for disk diffusion method was used to assess susceptibility to amikacin (AMK: 30 µg), ampicillin–sulbactam (SAM: 10/10µg), cefepime (FEP: 30 µg), cefotaxime (CTX: 30 µg), ceftazidime (CAZ: 30 µg), ceftriaxone (CRO: 30 µg), ciprofloxacin (CIP: 5µg), colistin (CST: 10 µg), doripenem (DOR: 10 µg), doxycycline (DOX: 30 µg), gentamycin (GEN: 10µg), imipenem (IPM: 10 µg), levofloxacin (LVX: 5µg), minocycline (MIN: 30 µg), netilmicin (NET: 30µg), piperacillin (PIP: 100 µg), polymyxin B (PB: 300 units), rifampicin (RIF:5 µg), tetracycline (TET: 30 µg), ticarcillin (TIC: 75 µg), ticarcillin/clavulanic acid (TIM: 75/10 µg), tigecycline (TGC: 15 µg), tobramycin (TOB: 10 µg), trimethoprim/sulfamethoxazole (SXT: 1.25/ 23.75 µg)(Mast Diagnostics, Bootle, UK). For tigecycline, the interpretation of zone diameters was done using the British Society for Antimicrobial Chemotherapy (BSAC) breakpoints) sensitive ≥ 24 mm, intermediate 20-23 mm, resistant ≤ 19 mm)[16]. During each test, *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control organisms to ensure accuracy of the antimicrobial susceptibility assays.

Determination of clonal type

International Clones (IC), the so-called European Clones (EC) or Sequence Group (SG), isolates were identified using two multiplex PCR sets amplifying group 1 (assigned as EC II), group 2 (assigned as EC I) and group 3 (assigned as EC III) alleles of outer-membrane protein A (*ompA*), chaperone–subunit usher E (*csuE*) and the intrinsic carbapenemase (bla_{OXA-51} like) genes were used as previously described[17].

Detection of Genes Encoding Virulence Factors

A new in-house multiplex PCR, referred to as tetraplex PCR (t-PCR) was developed and optimized as a rapid and effective method for the simultaneous detection of the genes that encode the most important putative virulence factors in clinical isolate of MDR *A. baumannii* (Fig. 1). The t-PCR was performed with the following PCR amplification conditions: 10 µl of Master Mix (Ampliqon, Denmark), 1 µl of each primer (SinaClon, Tehran, Iran) (Table 2) and 2µl of 100 pg of genomic DNA in 25 µl PCR reaction. The primers for the *ptk* and *eps*A genes were used at a concentration of 5 pmol, those for the *pld pga*B genes were used at 7.5 pmol. The cycling conditions consisted of an initial single cycle at 95°C for 5 min, followed by 35 cycles of melting at 94°C for 1 min, annealing at

57°C for 40 sec, and elongation at 72°C for 45 sec. A final and unique cycle at 72°C for 5 min was included. To verify the presence or absence of amplification products, 5 µl from each PCR products was electrophoresed on a 2% agarose gel. As in the single PCR assay, each reaction was performed in duplicate.

Statistical analysis

Statistical analysis was performed with the SPSS software (version 13, Chicago, IL, USA) package using chi-square and Fisher's exact tests.

RESULTS

Antimicrobial Susceptibility Testing

The antibiotic resistance profile of *A. baumannii* isolate from clinical sources is shown in Table 2. *Acinetobacter baumannii* strains were resistant (including intermediate susceptibility) to 7 to 21 antibiotics per isolate (median of 14) of 24 antibiotics; all isolates were resistant to cefepime, ceftriaxone, gentamycin and trimethoprim/ sulfamethoxazole while all the *A. baumannii* isolates were responsive to polymyxin B. Amongst all the selected antibiotics, rifampicin resistance rates showed significant difference between respiratory and wound isolates. 98% of respiratory vs 29% of wound isolates (p=0.001) of *A. baumannii* were resistant to rifampicin.

MDR and XDR phenotype proportion among isolates was 75% and 18%, respectively. Drug resistance pattern showed that 13% of the *A. baumannii* isolates were resistant to colistin. Nine colistin resistance *A. baumannii* (CRAB) isolates were from respiratory tract specimen, 3 were from operative wound specimen and one was from a patient with urinary tract infection (UTI). Thus, 9%, 3% and 1% of isolates from pneumonia, postoperative wound infection and UTI were resistant to colistin, respectively. Resistance to colistin was observed in sequence group 1 (7 patients), sequence group 2 (3 patients), and sequence group 5 (2 patients) and sequence group 6 (1 patients).

Nine (69%) and 2 (15.4%) colistin resistance *A. baumannii* isolates had MDR and XDR phenotype, respectively. Five (38.5%), 3 (23%) and 1 (7.5%) MDR isolates of CRAB were from pneumonia, postoperative wound infection and blood, respectively. The MDR trait was higher among *A. baumannii* isolates from pneumonia than isolates from postoperative wound infection and blood. Two XDR isolates of CRAB were from pneumonia. One CRAB isolates in this study, which was derived from urine specimen, had non-MDR phenotype. Eight percent of isolates showed resistance to colistin, rifampicin, and tigecycline (CRT) simultaneously. CRT Resistant varied at each of the specimens, 11.8% (6/51) and 7.5% (2/27) of respiratory and wound isolates showed CRT resistant antibiotype, respectively. Simultaneous resistance to CRT was observed in sequence group 1 (5 patients), sequence group 2 (2 patients), and sequence group 6 (1 patient). Six (75%) CRT resistance *A. baumannii* isolates had MDR phenotype. Five (83.3%) and one (16.6%) MDR isolates of CRT resistance *A. baumannii* were from pneumonia and postoperative wound infection, respectively. Interestingly, all of 13 CRAB resistance isolates were susceptible to tigecycline

Table 1. Primer sequences used in detection of genes encoding virulence factors

Gene	Primer sequence 5'-3'	Expected size of amplicon (bp)	Reference
ptk	GGCTGAGCATCCTGCAATGCGT ACTTCTGGAGAAGGGCCTGCAA	597	This study (In all primer sequences)
*pga*B	AAGAAAATGCCTGTGCCGACCA GCGAGACCTGCAAAGGGCTGAT	490	
*eps*A	TGCGAGTTGTGCAGTTACCTCCG GCCAGCTGCTTTATAGCGTCCCA	358	
pld	CGCGCGATATTTGCGGAAACGG CGCGGCTCGCTTAAGGCTGATT	253	

Table 2. Sources of 100 nonreplicate cultures with antimicrobial susceptibilities

Culture source	No. resistance to drugs																								
	Group A[a]								Group B												Group C				
	AMK[b]	CAZ	CIP	CRO	DOR	IPM	GEN	SAM	TOB	CTX	DOX	FEP	LVX	MIN	PIP	SXT	TET	TGC	TIC	TIM	CST	NET	PB	RIF
Respiratory tract (51)	43	51	36	51	46	43	51	37	46	51	21	51	48	11	51	51	38	9	42	36	6	33	6	50
Wound (27)	22	27	16	27	24	17	27	12	18	27	11	27	25	6	27	27	15	5	19	17	3	19	3	17
Other(22)	20	22	13	22	22	18	22	8	11	22	6	22	20	4	22	22	9	2	14	10	4	14	13	19
Total(100)	85	100	65	100	92	78	100	57	75	100	38	100	93	21	100	100	62	16	75	63	13	66	4	86

[a]; Considerations in the assignment of agents to Groups A, B and C include clinical efficacy, prevalence of resistance, minimizing emergence of resistance, cost, FDA clinical indications for usage, and current consensus recommendations for first-choice and alternative drugs. 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. Group B comprises agents that may warrant primary testing. Group C includes agents that have a clinical indication for the organism [25].

[b]; Abbreviations: CSF, cerebrospinal fluid; AMK, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; CRO, ceftriaxone; CST, colistin; CTX, cefotaxime; DOR, doripenem; DOX, doxycycline; FEP, cefepime; GEN, gentamycin; IPM, imipenem; MIN, minocycline; NET, netilmicin; LVX, levofloxacin; PB, polymyxin B; PIP, piperacillin; RIF, rifampicin; SAM, ampicillin/sulbactam; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; TGC, tigecycline; TIC, ticarcillin; TIM, ticarcillin/clavulanic acid; TOB, tobramycin .

and/or tobramycin.

Determination of clonal type

Based on two multiplex PCRs designed to detect allelic variations in the *ompA, csuE* and bla_{OXA-51} like genes, 5 different sequence groups (SG1,2,4-6) were identified among the 100 *A. baumannii* isolates (Fig. 2 and 3). Considerable number of the isolates (68%) belonged to SG1. Twenty-one (21%) isolates pertained to SG2. Eleven of the isolates (11%) belonged to 3 novel variant of SG4-6, defined according to new combination of amplified products obtained from the two separate multiplex PCRs that did not correspond to previously defined SG1, SG2 and SG3 (European clones II, I and III, respectively) (Table 3). *A. baumannii* isolates with SG1 clone were assigned to various specimens. In our study no SG3 was found in 100 isolates.

The lower respiratory tract was the most frequent site of isolation 35, 12, 3 and 1 of 100 patients for SG1, SG2, SG4 and SG6, respectively. Twenty isolates from wound swab were assigned to SG1, 6 were assigned to SG2, 1 assigned to SG5. SG1 was also isolated from 8 urine samples, 6 blood samples and 2 CSF samples , while SG2 was also isolated from the urine of 2 patients and one CSF sample and the new variant sequence groups was also isolated from 2 urine and 1 bloodstream samples. The11.7%, 14.3% and 18% of SG1, SG2 and novel SG_S were assigned to colistin resistant antibiotypes, respectively. According to the best of our knowledge, this is the first time that the prevalence of International Clones has been described in southwest of Iran.

Detection of Genes Encoding Virulence Factors

Generally, all of the isolates were positive for both the *pld* and the *pga*B virulence genes tested. The prevalence of the *ptk* and the *eps*A genes among of isolates differs noticeably in different clonal types. In the present study, the prevalence of *ptk* and *eps*A genes was 71% and 38%, respectively. Coexistence of *ptk* with epsA genes was observed in 29% of the isolates. *ptk* was also detected from 31 wound samples, 28 respiratory samples, 8 urine and 4 other samples, while *eps*A was also detected from the respiratory sample of 19 patients, 14 wound sample and 5 other samples, the coexistence of *ptk* and *eps*A was also detected from 15 respiratory samples, 11 wound sample and 3 other samples.

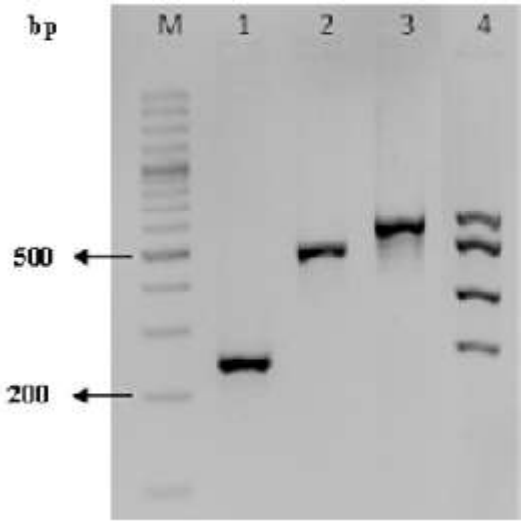

Fig. 1. Examples of multiplex PCRs designed to selectively amplify encoding PLD, PgaB, PTK and EpsA genes of *A. baumannii* isolates. Strains in lanes 1, 2 and 3 were *pld*, *pga*B and *ptk* positive, respectively. Strain in lanes 4 was *pld*, *pga*B, *ptk* and *eps*A positive. Lanes labelled M contain a 100-bp size ladder. A negative (water) control is contained in lane 5

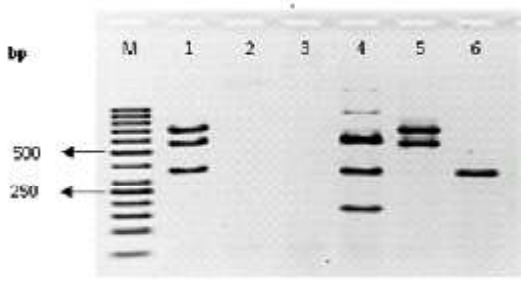

Fig. 2. Multiplex PCRs designed to selectively amplify *ompA, csuE* and bla_{OXA-51} like allele of standard strains belonging to Group 1, Group 2 and Group 3. All strains belonging to the Group 1 international clonal complex yielded all three fragments in the Group 1 PCR(lane 1), and none in the Group 2 PCR (lane 2), while strains belonging to Group 2 gave the expected converse results (lanes 3 and 4), despite some mismatches between the primer sequences. Strains belonging to Group 3 which gave the top two fragments (for the other two loci) in the Group 1 PCR (lane 5) and share the same *ompA* allele as Group 2, gave only the middle fragment (for *ompA*) in the Group 2 PCR (lane 6). Lane labeled M contain a size ladder

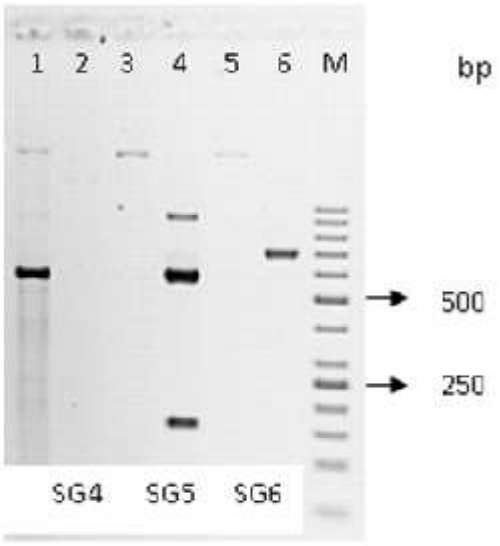

Fig. 3. Examples of multiplex PCRs designed to selectively amplify *ompA, csuE* and bla_{OXA-51} like alleles of isolates belonging to variant sequence groups that defined according to new combinations of amplified products obtained in the two separate multiplex PCRs that did not corresponding to previously defined sequence groups 1, 2 and 3. Lanes 1, 3, 6 yielded fragments in the Group 1 PCR and Lanes 2, 4, 5; yielded fragments in the Group 2 PCR. Lane labeled M contain a size ladder. The bottom of figure showing the variant sequence groups found in this study according to yielded fragments in the Group 1 and 2 PCRs.

DISCUSSION

The increasing reports of MDR *A. baumannii* indicate a significance public health concern[18]. Generally, tigecycline and colistin are used to treat MDR *A. baumannii* infections due to limited antibiotic choices. One study conducted on 6 hospitals in Iran during 2006-2007 showed that 8.8% of *A. baumannii* strains were resistant to tigecycline[19]. Another report conducted on Namazee hospital in Shiraz between 2007 and 2008 showed that the resistance rates of *A. baumannii* to colistin were 1.3%[6]. The present study revealed that 16% vs 8.8% and 13% vs 1.3% of *A. baumannii* isolates are resistant to tigecycline and colistin, respectively, by comparison with previous studies in Iran[19,6]. The tigecycline still is not widely used in the hospitals located in Iran. This may be one of

the main reasons for the lower rate of resistance to tigecycline found among MDR *A. baumannii* isolates obtained from the Iran hospitals.

The most prevalent (72.2%, 13/18) XDR *A. baumannii* strain contained a unique antimicrobial resistance profile (AMK, CAZ, CIP, CRO, CST, CTX, DOR, DOX, FEP, GEN, IPM, NET, LVX, PIP, RIF, SAM, SXT, TET, TIC, TIM, TOB). This profile also detected from two staff isolates (data not shown); suggesting horizontal transfer and study isolates were representative of the hospital-wide *A. baumannii* strains. We have shown here that resistance to imipenem increased from 18% to 78% (60% increases)[6]. Carbapenems, mainly imipenem and meropenem, have been used to treat MDR *A. baumannii* infections[18] and has been widely used in Iran in recent years, increasing number of imipenem-resistant isolates are emerging with the wide spread use of carbapenems.

Eight (61.5%) colistin-resistant isolates which were clonal types SG1 (5 isolates), SG2 (2 isolates), and SG6 (1 isolates), belonged to an antimicrobial resistant profiles: AMK, CAZ, CIP, CRO, CST, CTX, DOR, DOX, FEP, GEN, NET, LVX, PIP, RIF, SXT, TET, TGC, TIC, TIM. This may be due to the fact that in recent years the use of colistin in treatment of MDR *A. baumannii* infections is observed in Iran. MDR *A. baumannii* strains associated with community acquisition were excluded but there were epidemiological changes in the types of hospital-associated MDR *A. baumannii* strains in the ability over a period of time included in our study, with an associated change in antimicrobial resistance patterns.

MDR *A. baumannii* infections are associated with increased mortality attributed and due to the limited drug of choice for these infections, rifampicin-based treatments have been proposed as an alternative medication regimen[20]. In spite of the lack trials on rifampicin experience, up to 86% *A. baumannii* isolates identified in this study are resistant to rifampicin. In addition, this study is the first report the high prevalence of rifampicin-resistant MDR *A. baumannii* in Iran. The high resistance rate of MDR *A. baumannii* to rifampicin was also detected by Giamarellos-Bourboulis *et al.* [21]. The outbreak of MDR *A. baumannii* with resistance to rifampicin, tigecycline, and colistin is a critical concern that hospitals must control. Such epidemiological surveillance began to be used much earlier in the hospitals located in Iran, according to the alarming emergence of drug-resistant strains of *A. baumannii*.

This study demonstrates that the distribution of rifampicin-resistant MDR *A. baumannii* isolates varies between the samples. Namazee hospital receives more patients that are referred from nearby, smaller hospitals and clinics. Such relocations could result in the interhospital spread of resistant strains. In contrast, the intrahospital dissemination of MDR *A. baumannii* might account for the clonal spread of rifampicin–resistant isolates with different clonal-types in this hospital. This occurrence put emphasis on the importance of infection-control strategies for reduction of clonal spread within hospitals.

In the current study, the MDR *A. baumannii* isolates belonged to various clonal types that are widely distributed across various specimens as well as among all the age groups of patients (data not shown). This fact suggests that different MDR *A. baumannii* clone was circulated in the Namazee hospital.

The Detection of genes encoding virulence factors among different clonal types and clinical isolates of MDR *A. baumannii* has not been previously reported. All of the *A. baumannii* isolates, in this study, were positive for *pga*B gene. This result is in agreement with Choiet *et al*.[22] investigation showing the prevalence rate of this gene among the tested isolates. *A. baumannii pga*B that encodes a predicted 510-amino-acid protein with a polysaccharide deacetylase domain shares 33% identity with *E. coli* PgaB that is predicted to be an outer membrane lipoprotein which along with PgaA, is necessary for Poly-2-(1-6)-N-acetylglucosamine (PNAG) export[23].

In addition to its role as a major component of biofilms, PNAG is an important virulence factor which associated with surface and cell-to-cell adherence and protects bacteria against innate host immune system[22]. Choi *et al*.[22] showed that 47% (14/30) strains produced high levels of PNAG, 47% (14/30) strains produced low to moderate levels of PNAG, and 6% (2/30) strains did not synthesize detectable PNAG. This variable production of PNAG might reveal differences in regulation of gene expression and/or mutations in the *pga* loci.

In the current study, 100% of all the isolates were positive for the *pld* gene. The prevalence of the *pld* gene among the clinical isolate has not been previously reported. A transposon phospholipase D (PLD) gene (*pld*) mutant resulted in decreased *A. baumannii* epithelial cell invasion, the ability to proliferate in serum, bacteremia and colonization of visceral host organs in a murine infection model.

Although recent report has described the presentation of the *ptk* and *eps*A genes in *A. baumannii* that required for a capsule-positive phenotype remarkably, almost nothing is known about any aspect of the capsular polysaccharides produced by clinical isolate of this bacterial pathogen. In the present study, the prevalence of *ptk* and *eps*A genes was 71% and 38%, respectively. Coexistence of these genes was observed in 29% of the isolates. Further study is needed to clarify the expression the virulence factors and their regulation in clinical isolate of *A. baumannii*. Limitations of current study are the small sample size of clinical isolates.

In conclusion, the results of this study provide the first direct evidence of emerge and clonal spread of rifampicin-resistant MDR *A. baumannii* and clonal lineage within hospitals in southwest of Iran. The spread of rifampicin, tigecyline and colistin-resistant MDR *A. baumannii* strains via inter- and intrahospital turnover permits further investigations. We have shown here that the MDR *A. baumannii* strains indicate different clonal lineage patterns and repertoires of virulence genes profiles.

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Disclosure Statement

No competing financial interests exist.

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