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High Prevalence of Iron Acquisition Genes Among *Acinetobacter baumannii* Strains Isolated From Patients With Urinary Tract Infections in Southeast of Iran

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Abstract

Background: *Acinetobacter baumannii* is the most clinically prominent species of the *Acinetobacter* genus and is commonly found in hospital environments. In mammals, the iron element is virtually unavailable to invading bacteria, being mainly incorporated into iron transport and storage proteins. Therefore, iron acquisition systems are important factors for the pathogenicity of *A. baumannii* strains.
Objectives: The aim of this study was to determine the frequency of iron acquisition genes among *A. baumannii* isolates, collected from patients with urinary tract infections, for the first time in Iran.
Patients and Methods: A total of 100 *A. baumannii* isolates were collected from patients with urinary tract infections in Zabol, southeast of Iran. All isolates were evaluated to determine the prevalence of iron acquisition genes, including *tonB* (TonB-dependent receptor), *barA* (acinetobactin ABC transporter), *feoB* (ferrous iron transport protein B), *entA* (acinetobactin siderophore precursor), *A1S_2563* (siderophore-interacting protein), and *hemO* (heme oxygenase) using the multiplex polymerase chain reaction (PCR) method.
Results: A high prevalence of genes encoding iron acquisition systems were observed in *A. baumannii* isolates. The frequency of *tonB*, *barA*, *feoB*, *entA*, *A1S_2563,* and *hemO* genes were 85, 97, 99, 98, 99, and 95%, respectively. Based on the distribution of the various iron acquisition genes, all the studied isolates exhibited seven gene profile patterns.
Conclusions: This is the first report on the prevalence of iron acquisition genes among *A. baumannii* isolates collected from patients with urinary tract infections. The high prevalence of iron acquisition genes in *A. baumannii* isolates suggests that these virulence factors play an important role in the development of urinary tract infections.

Keywords: Virulence, Iron, Urinary Tract Infections, Multiplex Polymerase Chain Reaction, *Acinetobacter baumannii*

1. Background

Acinetobacter baumannii is a Gram-negative bacterial pathogen that has appeared globally as a serious risk to human health and is commonly found in hospital environments (1). This nosocomial pathogen typically causes severe infections, including urinary tract infections (UTI), pneumonia, bacteremia, meningitis, and wound-associated infections (2). *Acinetobacter baumannii* strains must compete with the host for essential nutrients during colonization. Iron is an essential nutrient for many cellular biochemical pathways. In the mammalian host, low free-Fe concentration portends a non-specific host defense mechanism against infection; *A. baumannii* strains use this as a stimulus to express active iron-acquisition systems. Iron acquisition mechanisms help the bacteria survive in adverse environmental conditions and facilitate the development of an infection (3-5).

Siderophore biosynthesis is essential for the virulence of many important Gram-negative pathogens. In response to low iron, *A. baumannii* produces siderophore acinetobactin to acquire this essential micronutrient (6). Several iron acquisition components in *A. baumannii* strains are thought to be associated with its pathogenicity, including acinetobactin siderophore precursor (EntA) (7), siderophore-interacting protein (A1S_2563) (8), TonB-dependent receptor (TonB) (9), ABC transporter involved in the secretion of acinetobactin (BarA) (6), ferrous iron transport protein B (FeoB) (10) and heme oxygenase (HemO) (10).

To date, several studies have reported on the epidemiology, resistance profiles and virulence factors of *A. baumannii* isolates from Iranian patients (11-18). To our knowledge, the prevalence of iron acquisition genes, as an important virulence factor of *A. baumannii*

strains isolated from patients with UTIs, are unknown in Iran. In addition, the distribution of genes coding iron acquisition systems, including *tonB*, *barA*, *feoB*, *entA*, *A1S_2563,* and *hemO* in clinical isolates of *A. baumannii*, has never been reported in different regions of the world.

2. Objectives

This study was carried out for detection of iron acquisition genes, including *tonB*, *barA*, *feoB*, *entA*, *A1S_2563,* and *hemO* genes of *A. baumannii* strains isolated from patients with UTIs in Zabol, southeast of Iran.

3. Patients and Methods

3.1. Study Population and Sample Collection

This cross-sectional study was conducted on 183 volunteer patients attending two major hospitals of Zabol, Iran, between January and July 2014. The inclusion criterion was attendance to hospitals for symptoms of lower or upper urinary tract infections. The exclusion criterion was receiving any antibiotic therapy within one week before sampling. In this study, all of patients provided consent for use of their sample to determine prevalence of iron acquisition genes in *A. baumannii* isolates.

3.2. Bacterial Isolates

Specimens were taken from clean-catch sample, midstream urine and urinary catheters. All samples were immediately transferred on ice to the microbiology laboratory. The identification of *A. baumannii* was performed using conventional bacteriological methods such as oxidase, triple sugar-iron (TSI), sulfide-indole motility (SIM), hemolysis on sheep blood agar, and growth at 43˚C (19, 20). Isolates were considered as *A. baumannii* if they were Gram negative coccobacilli, non-fermentative, citrate positive, indole negative, TSI base/base, H_2S negative, urease negative, oxidase negative, catalase positive, and non-motile (17).

3.3. DNA Extraction

DNA template preparation was performed by the boiling method. Briefly, *A. baumannii* isolates were grown overnight (16 hours) in 5 mL Luria Bertani (LB) broth (Merck, Germany) at 37˚C. Two milliliters of bacterial isolates were then pelleted and resuspended in 200 µL of sterile double-distilled water. The cells were lysed by heating at 95˚C for 10 minutes. After centrifugation (at 12000 rpm for five minutes), the supernatants were stored as a DNA template at -20˚C until used for multiplex PCR.

3.4. Multiplex-Polymerase Chain Reaction (PCR) Method for Detection of Iron Acquisition Genes

A new Multiplex PCR was developed and optimized as a rapid and effective method for the simultaneous detection of the genes that encode the iron acquisition systems in UTI-causing isolates of *A. baumannii*. Specific primers (Table 1) were designed to amplify iron acquisition genes in *A. baumannii* isolates by using MPprimer and an online software (http://biocompute.bmi.ac.cn/MPprimer/run_example.htmL). Multiplex PCR was performed in a reaction mixture with a total volume of 25 µL, containing 12.5 µL of 2 × MasterMix red Taq polymerase (Ampliqon, Pishgam, Iran) and 0.2 µM /µL of each primer (forward and reverse primer each one, 1 µL) (Pishgam, Iran), 3 µL (approximately 100 ng/µL) of genomic DNA, and 7.5 µL dd H_2O. The Multiplex PCR procedure was as follows: initial denaturation at 94˚C for five minutes, followed by 30 cycles consisting of denaturation (94˚C for 30 seconds), annealing (62˚C for 50 seconds) and extension (72˚C for 70 seconds), followed by a final extension step at 72˚C for five minutes. Amplification was performed using a gradient Eppendorf Mastercycler® pro (Eppendorf, Germany). The Multiplex PCR products were evident after migration by gel electrophoresis (120 V/208 mA) on 2% agarose gel prepared with tris-acetate-EDTA (TAE) 1X (0.1 M tris, 0.09 M boric acid and 1 mM EDTA), stained with ethidium bromide 0.5 µg/mL, visualized by UV and photographed with a Polaroid camera. A 100 bp DNA ladder (Fermentase, 100 bp) was used as a size standard.

4. Results

4.1. Prevalence of Iron Acquisition Genes

In total, 100 isolates of *A. baumannii* were identified by microbiology standard methods. Generally, all of the isolates were positive for the tested iron acquisition genes (Figure 1). A high prevalence of genes encoding iron acquisition systems in *A. baumannii* isolates was observed. The prevalence of the *feoB* and the *A1S_2563* genes amongst *A. baumannii* isolates were noticeably high (99%). The Multiplex PCR results showed that among the 100 *A. baumannii* isolates, 98 were positive for *entA*, 97 for *barA*, 95 for *hemO*, and 85 for *tonB* iron acquisition genes. Seven different gene profile patterns were identified among *A. baumannii* isolates, referred to as Ab (Table 2). Ab1 was determined by the presence of all of the studied genes, and was the most identified pattern, found in 80 isolates. The association of five genes was recognized in Ab2, Ab3 and Ab4 patterns (17 isolates). Two patterns, Ab5 and Ab6, were represented by strains possessing a combination of four iron acquisition genes (2 isolates). One *A. baumannii* isolate (Ab8) lacked all tested iron acquisition genes (Table 2).

Table 1. Primer Sequences Used in Detection of Iron Acquisition Genes of *Acinetobacter Baumannii* Isolates[a]

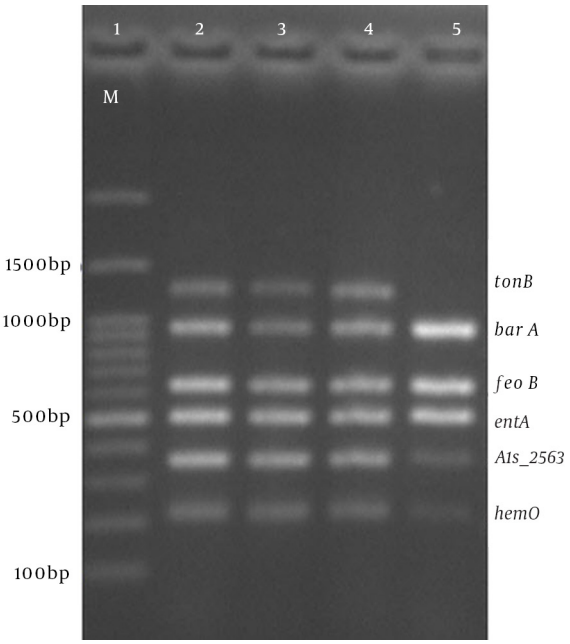
Genes	Primer Sequences (5′ → 3′)	Size, bp	Tm, ˚C
entA	F-ATCGTGGTCACAGGTGCTGCAA	504	64
	R-AGGGGCGATTTCAAGTGCCAGA		
feoB	F-AAGTCGCCAACTATGCCGGTGT	636	64
	R-AAGGCGCTGCCCATGCAAAAAC		
hemO	F-TCGTGGCCGCTCAAAACAAGCA	215	64
	R-AGGCCGCTAAATTACGTGCAGC		
barA	F-TATGCGCGCGGCATTCGTGATA	959	64
	R-TCGGCATGAGCAGCTAAGGCAA		
A1S_2563	F-TGCGCATGGCTCAAATGGGGAA	357	64
	R-TGACTGCCTTGCTCATGCACAG		
tonB	F-TTGTGGTGCCTCTGCAATCGGT	1279	64
	R-TCGTGTACCCAAACGAGCAGGA		

[a]The references for the above table are the present study.

Table 2. Iron Acquisition Gene Patterns Identified Amongst the Studied Isolates

Pattern	*tonB*	*barA*	*feoB*	*entA*	*A1S_2563*	*hemO*	No. Strains
Ab1	+	+	+	+	+	+	80
Ab2	-	+	+	+	+	+	12
Ab3	+	+	+	+	+	-	4
Ab4	+	-	+	+	+	+	1
Ab5	-	-	+	+	+	+	1
Ab6	-	+	+	-	+	+	1
Ab7	-	-	-	-	-	-	1
Total	85	97	99	98	99	95	100

Figure 1. Iron Acquisition Gene Profiles Obtained by Multiplex Polymerase Chain Reaction



Lanes: 1, 100 pb DNA Molecular size marker; 2-4, *tonB*, *barA*, f*eoB*, *entA*, *A1S_2563* and *hemO* genes; 5, *barA*, *feoB*, *entA*, *A1S_2563* and *hemO* genes.

5. Discussion

We assessed the utility of the multiplex PCR for rapid detection of the six iron acquisition genes among *A. baumannii* strains isolated from patients with UTIs. In the present study, the frequency rate of the *entA* gene encoding for acinetobactin siderophore precursor was 98% among the studied isolates. This gene, which is essential for the biosynthesis of the acinetobactin precursor, 2,3-dihydroxybenzoic acid (DHBA), is located outside of the acinetobactin gene cluster, which otherwise harbors all genes needed for acinetobactin biosynthesis, export and transport. Previously, experimental infections revealed the role of DHBA and acinetobactin intermediates in siderophore production, iron acquisition and virulence of the *A. baumannii* ATCC 19606T strain (7). We observed a high prevalence rate (97%) of *barA* gene among *A. baumannii* isolates of urine patients with UTIs in Zabol, southeast of Iran. The *bar* gene that is needed for the secretion of acinetobactin is located at a 26.5-kb chromosomal region, harboring seven operons (6). Acinetobactin siderophore that is produced by *A. baumannii* strains is secreted through a siderophore efflux system of the ABC superfamily, consisting of proteins encoded by *barA* and *barB* genes (6, 7). However, so far there are no reports about the prevalence of *entA* and *barA* genes among *A. baumannii* isolates. Our results indicated that the prevalence rate of *A1S_2563* gene was 99% among studied

A. baumannii isolates. Siderophore gene cluster II contains 15 genes involved in siderophore biosynthesis (A1S_2567-2581), three genes involved in the recognition and uptake of the ferric siderophore; *A1S_2563* gene is one of them (8). Also, *A. baumannii* strains contain 8 to 22 predicted *TonB*-dependent receptors in their genome that are involved in acinetobactin transport (9). In this study, the frequency rate of *tonB* gene was 85% among studied *A. baumannii* isolates. A previous study showed that the expression and function of each *A. baumannii* TonB system is variable (9). Furthermore, in our study the prevalence of *feoB* and *hemO* genes was 99% and 95%, respectively. *Acinetobacter baumannii* encodes ferrous iron uptake systems, most notably FeoAB transporters, which are required for iron acquisition and pathogenesis. FeoAB system with its regulator FeoC and at least one FeoB, has been identified in all sequenced strains along with a FeoA and FeoC (10). The *hemO* gene is involved in oxidative cleavage of heme to release iron and this gene is located inside the heme utilization gene cluster in genome of *A. baumannii* strains (10). However, according to published data, there is no information on the prevalence of, *A1S_2563*, *tonB*, *feoB*, and *hemO* genes in *A. baumannii* isolates. On the other hand, the analysis of the relationship between the presences of different combinations of iron acquisition genes among *A. baumannii* isolates, allowed us to separate tested isolates into seven gene profile patterns, Ab1 to 7 (Table 2). The current study showed that 80% of *A. baumannii* isolates carried all the studied iron acquisition genes. Also, 98% of isolates were associated with three genes, including *feoB*, *entA* and *A1S_2563* genes (Ab1- Ab5). The main new finding of this study was the high prevalence genes encoding the major iron acquisition systems among *A. baumannii* in Zabol, Southeast of Iran. Presumably, geographical differences, the levels of public health, hospital's health and even methods of sampling have important roles in the prevalence rate of iron acquisition genes in various *A. baumannii* isolates.

In conclusion, this is the first study that was designed for determining the frequency of iron acquisition genes among *A. baumannii* isolates collected from patients with urinary tract infections. The high prevalence of iron acquisition genes in these isolates suggests that the products of these genes can play an important role in the development of UTIs. Therefore, further studies worldwide are needed to better understand the prevalence rate and the interaction of different iron acquisition genes among different clinical *A. baumannii* isolates.

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Footnotes

Authors' Contribution: Hossein Ali Abdi, Bahman Hormozi and Nafiseh Noorzehi: sampling, processing and performing of conventional and molecular procedures, data collection and data interpretation; Mohsen Najimi: study design, management, advising and supervision; Hossein Ali Abdi: funds collection, genetic analysis, design of primers, literature review and manuscript preparation.

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