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Original Article

Determining the Prevalence and Detection of the Most Prevalent Virulence Genes in *Acinetobacter Baumannii* Isolated From Hospital Infections

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ABSTRACT

Background and Aims: *Acinetobacter baumannii* is mostly a cause of septicaemia, pneumonia and urinary tract infections following hospitalization of patients with more severe illnesses. The aim of this study was to determine the prevalence and detection of the most prevalent virulence genes in *A. baumannii* isolated from hospital infections of two largest hospitals of Tehran, Iran.

Materials and Methods: In this cross-sectional study, 500 clinical specimens were obtained from various types of hospital infections over a period of 6 month, consisting of blood (98 samples), phlegm (141), urine (92), pus (134) and cerebrospinal fluid (35) from patients admitted to the Payambaran and Baqiyatallah Hospitals in Tehran. The isolated organisms were identified based on colony morphology, microscopic characteristics and various biochemical tests according to some standard laboratory methods. Conventional polymerase chain reaction (PCR) was employed to confirm the identity of the isolates.

Results: *A. baumannii* was isolated from 121 (24.2%) of the 500 cultured samples. The highest isolation of *A. baumannii* was observed in blood samples while cerebrospinal fluid had the least. The isolation rate recorded for blood samples in this study was 43.87%. fimH gene was the most virulence gene detected in 74% of samples, sfa/focDE and afa/draBC genes were next highly detected genes, respectively. The lowest isolations were observed in PapG III, papC and PapG II genes.

Conclusions: High prevalence of *A. baumannii* and their virulence genes showed hospital prevalence of these bacteria, thereby causing many problems for infections control and treatment. Therefore, determining this bacterium by molecular methods and designing conservation programs for the control of different infections in parts of the hospital seems to be urgently needed.

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Introductison

Genus Acinetobacter is comprised of a group of Gram-negative, oxidase-negative, catalase positive, non-motile, non-spore forming and strictly aerobic cocobacilli, which are widely distributed in nature [1, 2]. Acinetobacter genus consists of 25 validly-named species and 9 genomic species defined by genomic DNA–DNA hybridization [3]. Four species of this genus (*A. calcoaceticus*, *A. baumannii*, *A. pittii and A. nosocomialis*) are often referred to as *A. calcoaceticus-A. baumannii* complex, form a closely related group of glucose-acidifying strains which are difficult to distinguish from each other by phenotypic tests [4, 5]. *A.baumannii* is one of the six ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and Enterobacter species) bacteria of clinical importance [6].It is recognized as an important human pathogen causing severe infections in hospitalized patients as well as deadly cases of community acquired pneumonia [7-9]. Surveillance studies have identified *A. baumannii* as the fifth cause of pneumonia, after *P.aeruginosa*, in hospitalized patients, especially in intensive care units [10]. This organism was reported to be the fifth most common pathogen involved in infections in intensive care units among 75 countries of the five continents [11]. This bacterium has also been associated with cases of septicemia, endocarditis, meningitis, skin and soft tissue infections, wound, respiratory tract and urinary tract infections [12, 13]. *A. baumannii* was a predominant isolate from wounded soldiers serving in Iraq [14].

The emergence and global dissemination of *A. baumannii* is attributed to its characteristics such as a remarkable ability to survive desiccation and persist for prolonged periods throughout the hospital environment [15-18], high rate of nosocomial transmission [18-20], and an inherent ability to acquire antibiotic resistance genes [21,22]. Prevalence of Virulence Factors (VF) is contributed to pathogenesis in bacteria [23]. Virulence factors help bacteria to colonize on the epithelium, evade and inhibit the host's immune response through biofilm formation, and obtain nutrition from the host [24, 25]. During the past decades, new VF have been described in Escherichia coli. Pathogenicity-associated islands (PAI) are blocks of VF genes that provide a mechanism to coordinate horizontal transfer of VF genes between lineages, and even between species, and have emerged as characteristic of diverse pathogenic bacteria, including uropathogenic *E. coli* strains [26]. Recognized or determine VF in uropathogenic E. coli include diverse adhesins, as P fimbriae (pap genes), S and F1C (sfa), Drantigen family (afa/dra), type 1 fimbriae (fimH) [26, 27] and curli fibers (csg) [28]; fibronectin receptor (fbn) [29]; toxins, as cytotoxic necrotizing factor (cnf) [30]; siderophores, as yersiniabactin (fyuA) and aerobactin (iutA); invasins as IbeA; polysaccharide coatings as group II and III capsules (kpsMT); serum resistance (traT) and colicin V production (cvaC) [31]. Some of the virulence factors identified in *A. baumannii* included fimbriae and/or capsular polysaccharide [32, 33], the polysaccharide capsule, [34], siderophores [35, 36], biofilm [37-40], Phospholipase D and Phospholipase C [41]. However, as pointed out by Cerqueira and Peleg, [42], very little information is known about the virulence factors in *A. baumannii* and identification of these factors could contribute to the development of novel therapeutic alternatives for the control of clinically relevant pathogen. Adhesive virulence factors are divided into two subgroup; Fimbrial VF which colonization is related to this subgroup: P fimbriae (pap genes), S (sfa/focDE), Dr antigen family (afa/draBC), type I fimbriae (fimH) and non-fimbrial VF: curli fibers (csgA); fibronectin receptor (fnb); polysaccharide coatings as group II capsules (kpsMT) [43].

Identification of virulence factors in *A. baumannii* is a key to fighting this pathogen so the aim of the present study was to determine the isolation rate of *A. baumannii* in human clinical samples in Tehran, Iran and to assess the *A. baumannii* isolates for genes coding for virulence factors.

Materials and Methods

Source of bacteria

In a cross- sectional study conducted over a period of 6 months (from September 2012 to March 2013), 500 clinical specimens comprised of blood (98 samples), phlegm (141), urine (92), pus (134), cerebrospinal fluid (35) were collected from patients admitted to the Payambaran and Baqiyatallah Hospitals in Tehran. The specimens were collected by a laboratory technician, properly labeled and transferred immediately to the microbiology laboratory. Medical Ethics Committee of Islamic Azad University of Shahrekord branch approved the research. All samples were taken from volunteer patients for this research. All ethical issues were considered and this research was performed with hospitals' permission.

Isolation and identification of *A. baumannii*

Each sample was streaked on blood agar (Merck, Germany) and MacConkey agar (Merck, Germany) and incubated aerobically at 37ºC for 24 hours. Non-hemolytic, opaque and creamy colonies on blood agar and non-lactose fermenting colonies on MacConkey agar were further sub-cultured on MacConkey agar and incubated for another 24 hours at 37ºC to obtained pure colonies. The isolated organisms were identified based on colonial and microscopic characteristics and various biochemical tests according to standard laboratory methods [44]. Stock cultures were maintained in both agar slant and 20% sterile buffered glycerin and were kept at -70°C. Genomic DNA was extracted from the bacterial isolates using the Genomic DNA Purification Kit (Merck, Germany) method. Conventional polymerase chain reaction (PCR) was employed to confirm the identity of the isolates. The reverse and forward primers (Cinagen, Iran) and size of product as previously published [45] were used for the detection of *A. baumannii* 16S-23S ribosomal DNA. The standard strains of *Escherichia coli* ATCC 25922 and *A. baumannii* ATCC 19606 were used for quality control purposes as the negative and positive controls respectively.

Detection of genes coding for virulence factors

Using multiplex PCR (Master Cycler Gradient, Eppendorf, Germany), the *A. baumannii* isolates were investigated for genes coding for some recognized virulence factors identified in uropathogenic *E. coli* strains. The virulence genes investigated in this study are presented in table 1. PCR programs (temperature and volume) for detection of 16S-23S ribosomal DNA and virulence factors genes in *A. baumannii* are summarized in table 2. The PCR amplified products (10μL) were subjected to electrophoresis in a 1.5% agarose (Fermentas, Germany) gel in 1X TBE buffer (Fermentas, Germany) at 80V for 30 minutes stained with solution of ethidium bromide and examined under ultra violet illumination (Uvi tec, England). The 100-bp ladder (Fermentas, Germany) was used as standard for determining molecular mass of PCR products.

Table 1. Primers used for detection of virulence genes in *A. baumannii*

Gene	Primer	Primer Sequence (5'-3')	Size of product (bp)	Reference
afa/draBC	afa1	GCTGGGCAGCAAACTGATAACTCTC	750	27
	afa2	CATCAAGCTGTTTGTTCGTCCGCCG		
cnf1	cnf1	AAGATGGAGTTTCCTATGCAGGAG	498	46
	cnf2	CATTCAGAGTCCTGCCCTCATTATT		
cnf2	cnf2a	AATCTAATTAAAGAGAAC	543	47
	cnf2b	CATGCTTTGTATATCTA		
csgA	M464	ACTCTGACTTGACTATTACC	200	47
	M465	AGATGCAGTCTGGTCAAC		
cvaC	ColV-CF	CACACACAAACGGGAGCTGTT	680	26
	ColV-CR	CTTCCCGCAGCATAGTTCCAT		
fimH	FimH F	TGCAGAACGGATAAGCCGTGG	508	26
	FimH R	GCAGTCACCTGCCCTCCGGTA		
fyuA	FyuA f	TGATTAACCCCGCGACGGGAA	880	[26,48]
	FyuA R	CGCAGTAGGCACGATGTTGTA		
ibeA	ibe10 F	AGGCAGGTGTGCGCCGCGTAC	170	[26,49]
	fibe10 R	TGGTGCTCCGGCAAACCATGC		
iutA	AerJ F	GGCTGGACATCATGGGAACTGG	300	[50]
	AerJ R	CGTCGGGAACGGGTAGAATCG		
***kpsMT* II**	kpsII F	GCGCATTTGCTGATACTGTTG	272	[26]
	kpsII R	CATCCAGACGATAAGCATGAGCA		
PAI	RPAi F	GGACATCCTGTTACAGCGCGCA	930	[26]
	RPAi R	TCGCCACCAATCACAGCCGAAC		
papC	pap1	GACGGCTGTACTGCAGGGTGTGGCG	328	[27]
	pap2	ATATCCTTTCTGCAGGGATGCAATA		
***PapG* II, III**	pGf	CTGTAATTACGGAAGTGATTTCTG	1070	[51]
	pGr	ACTATCCGGCTCCGGATAAACCAT		
sfa/focDE	sfa1	CTCCGGAGAACTGGGTGCATCTTAC	410	[27]
	sfa2	CGGAGGAGTAATTACAAACCTGGCA		
traT	TraT F	GGTGTGGTGCGATGAGCACAG	290	[26]
	TraT R	CACGGTTCAGCCATCCCTGAG		
A. baumannii	16S-23S	(F) CATTATCACGGTAATTAGTG	208	[45]
detection	ribosomal DNA	(R) AGAGCACTGTGCACTTAAG		

Table 2. PCR conditions for detection of virulence genes in *A. baumannii*

Gene	PCR program	PCR volume (50 µL)
afa/draBC, cnf1, csgA, cvaC, iutA, fyuA	1 cycle: 95 ^{0C} ------------ 4 min. 30 cycle: 95 ^{0C} ------------ 50 s 58 ^{0C} ------------ 60 s 72 ^{0C} ------------ 45 s 1 cycle: 72 ^{0C} ------------ 8 min	**5 µL PCR buffer 10X** **1.5 mM $Mgcl_2$** **200 µM dNTP (Fermentas)** **0.5 µM of each primers F & R** **1.25 U Taq DNA polymerase (Fermentas)** **2.5 µL DNA template**
cnf2, kpsMT II, *PAI, papC*	1 cycle: 94 ^{0C} ------------ 6 min. 34 cycle: 95 ^{0C} ------------ 50 s 58 ^{0C} ------------ 70 s 72 ^{0C} ------------ 55 s 1 cycle: 72 ^{0C} ------------ 10 min	**5 µL PCR buffer 10X** **2 mM $Mgcl_2$** **150 µM dNTP (Fermentas)** **0.75 µM of each primers F & R** **1.5 U Taq DNA polymerase (Fermentas)** **3 µL DNA template**
fimH, ibeA, PapG II-III, *sfa/focDE, traT*	1 cycle: 95 ^{0C} ------------ 4 min. 34 cycle: 94 ^{0C} ------------ 60 s 56 ^{0C} ------------ 45 s 72 ^{0C} ------------ 60 s 1 cycle: 72 ^{0C} ------------ 10 min	**5 µL PCR buffer 10X** **2 mM $Mgcl_2$** **200 µM dNTP (Fermentas)** **0.5 µM of each primers F & R** **1.5 U Taq DNA polymerase (Fermentas)** **5 µL DNA template**
16S-23S ribosomal DNA	**1 cycle:** **94 ^{0C} ------------ 6 min.** **30 cycle:** **95 ^{0C} ------------ 60 s** **58 ^{0C} ------------ 60 s** **72 ^{0C} ------------ 40 s** **1 cycle:** **72 ^{0C} ------------ 5 min**	**5 µL PCR buffer 10X** **2mM $Mgcl_2$** **150 µM dNTP (Fermentas)** **1 µM of each primers F & R** **1 U Taq DNA polymerase (Fermentas)** **3 µL DNA template**

Statistical analysis

The data were analyzed using SPSS version 16 (SPSS Inc, Chicago, IL, USA). Chi-square and Fisher's exact tests were used to identify statistically significant relationships between the following: source of bacterial isolates and distribution of virulence genes, of the *Acinetobacter baumannii* strains isolated from infected patients. $P< 0.05$ was considered statistically significant.

Results

Acinetobacter baumannii was isolated from 121 (24.2%) of the 500 samples cultured. The isolation rates from the various clinical samples are presented in table 3.

Table 3. Isolation rate of *A. baumannii* strains from human clinical samples

Clinical Samples	Samples (No)	*A. baumannii* (No)	Isolation rate
Blood	98	43	43.87
Phlegm	141	34	24.11
Urine	92	22	23.91
Pus	134	16	11.94
CSF	35	6	17.14
Total	500	121	24.20

Distribution of virulence genes in *Acinetobacter baumannii* strains isolated from clinical samples in human are shown in table 4.

Table 4. Distribution of virulence genes in *A. baumannii* strains isolated from clinical samples

Sample	*afa/draBC*	*cnf1*	*cnf2*	*csgA*	*cvaC*	*iutA*	*fyuA*	*fimH*	*kpsMTII*	*PAI*	*ibeA*	*papC*	*PapGII*	*PapGIII*	*sfa/focDE*	*traT*
Blood (43)	21	16	8	9	14	17	20	38	4	5	2	1	3	2	17	4
Phlegm(34)	19	10	12	-	4	-	19	28	6	-	-	5	6	2	18	2
Urine (22)	8	3	2	4	-	3	-	20	4	8	9	1	-	-	20	6
Pus (16)	10	11	7	2	8	1	2	2	-	2	-	-	-	-	3	-
CSF (6)	2	3	2	-	-	2	-	2	3	-	4	2	1	1	4	2
Total (121)	52	43	31	15	26	23	41	90	17	15	15	9	10	5	62	14
Percent	42.97	35.53	25.61	12.39	21.48	19.00	33.88	74.38	14.04	12.39	12.39	7.43	8.26	4.13	51.23	11.57

fimH gene was the most virulence gene detected in 47% of samples and follow that *sfa/focDE* genes and *afa/draBC* respectively were high detected. Lowest isolations were on *PapG* III, *papC* and *PapG* II gens.

Discussion

Nowadays controlling infections caused by gram negative pathogen bacteria such as *A. baumannii* and appearance of resistant isolates has become a clinical challenge [43]. We tested *A. baumannii* strains, isolated from various clinical samples, for the presence of genes that codify for various virulence factors by PCR, as described for uropathogenic *E. coli*. The 24.2% isolation rate reported in the present study is higher than the 9.4% reported by Jaggi et al. [52] from various clinical samples in a tertiary care hospital in India and the 11% reported by Siau et al. [53] in Hong Kong. The highest isolation of *A. baumannii* was observed from blood samples while cerebrospinal fluid had the least. Among the three different clinical specimens processed by Shanthi and Sekar [54], blood had the least number of *A. baumannii* isolates. The 43.87% isolation rate recorded for blood samples in this study is higher than the 23.8% reported by Jaggi et al. [52]. *A. baumannii* infections are extremely difficult to treat [55, 56] and the isolation of this organism from the various clinical specimens in this study was a cause for concern. Rahbar and Hajia [57] reported that *A. baumannii* organism is the most frequent cause of respiratory tract infections and they have isolated strains from 3-5% of patients with nosocomial pneumonia. In another study done by Rahbar et al. [58], a total of 88 strains of *A. baumannii* were isolated from clinical specimens obtained from patients hospitalized in an Iranian 1000-bed tertiary care hospital. The majority of isolates were from respiratory tract specimens. However, in this study the highest isolation of *A. baumannii* was observed from blood samples while cerebrospinal fluid had the least. Lautenbach et al. [59] reported annual prevalence of isolates from 0% to 21% of imipenem resistance *A. baumannii* from 1989 through 2004 while moreover there were 386 patients with *A. baumannii* isolates and during the period from 2001 through 2006. Marchaim et al. [60] reported infection rate of 17% of patients in a study carried out over 42 months. Maragakis et al. [61] observed occurrences that have been traced to common-source contamination (predominantly unhygienic respiratory and ventilator equipment), and to cross-infection by the hands of health care workers caring for infected patients. The same sources of infection can be responsible for the observed prevalence in this study. Furthermore, endemicity of several strains can be a result of a single endemic strain dominating at any given period [62].

We found that *fimH* gene was the most virulence gene and follow that *sfa/focDE* and *afa/draBC* genes respectively were high detected. Adherence is related to adhesins filamentous (fimbrial structures) or non-filamentous [63]. Fimbrial structures and their participation in adhesive properties involving fibronectin were investigated by means of agglutination assays [64]. A total of 104 clinical isolates of *A. baumannii* were collected from 3 hospitals in Kermanshah (Iran) showed they had two adhesive virulence factors of fimbriae *csg*A and *fim*H in 27 (54%) and 30 (60%) of cases, respectively [65]. Adhesive virulence factors are considered an important factor in adhesion, biofilm formation and survival of most bacteria and their virulence in human [23]. Compared to other gram-negative pathogens, relatively few virulence factors have been identified for *A. baumannii* [66]. Therefore, it can be concluded that the genes of *csg*A and *fim*H involved in forming biofilm [67, 68]. The existence of thin fimbriae in *A. baumannii* which are a major factor in adherence was described by Rosenberg *et al*. (1982) and Braun and Vidotto (2004) [33, 43].

Contrary to our study, none of Braun's isolates had relevant adhesive genes [43]. It gave that most of the samples in our study were isolated from blood, phlegm, urine, pus, cerebrospinal fluid; the difference between these two studies can be attributed to difference in the source of isolates and mechanism of adhesion in bacteria causing urinary tract infection**s** with those of other infections. Studies show that 30% of *A. baumannii* produce capsule [69] while we did not find the relevant gene (*kps*MT).

Conclusion

Acinetobacter baumannii was isolated from 24.2% of the samples cultured. The isolation of *A. baumannii* in this study is great concern because it is the most resistant of the genospecies and has the most clinical significance and is the mainly frequently detected species and is usually associated with outbreaks in the hospital locale.

Conflict of Interest

The authors declare that they have no conflict of interest

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