

Molecular Epidemiological Analysis of *Acinetobacter baumannii* Strains Isolated in Patients with Burn Injury

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Recently, there is a steady increase of infections caused by *Acinetobacter spp.*, especially *A. baumannii* in patients with burn injury. Hospital populations of microorganisms always include highly virulent and antibiotic-resistant strains. The analysis using molecular markers enables to estimate the epidemiologic relation between strains, reveal the presence of hospital strains, and identify the infection source.

The aim of the investigation was to assess the nature of the spread of significant epidemic strains *Acinetobacter baumannii* in burn patients, and identify a predominant genotype to determine the presence of hospital strains using molecular genetic techniques (polymerase chain reaction (PCR)).

Materials and Methods. By means of PCR we investigated some major virulence factors and antibiotic resistance of 60 of *A. baumannii* strains being isolated for more than two years from the burn unit patients. The antibiotic resistance of genes and virulence factors of *OXA-23*, *ISAbal*, *csuE*, *tonB* were studied. The polymorphism of genotypes was evaluated using χ -square test (Raymond M., Rousset F., 1995).

Results. The strains under study were revealed to have a high resistance level to carbapenems, *csuE* gene was identified in 40% strains, the gene being one of the film formation susceptibility factors of bacteria. *tonB* gene encoding the bacteria properties enabling to cause bacteremia promptly was isolated in 15% cases. In the genes encoding antibiotic resistance and virulent factors, we revealed a slight genetic differentiation level of strains (total, 9 combined genotypes with three genotypes predominating: A — 50% cases, F and I — 10% cases) that indicates both exogenous nosocomial infection of patients, and the presence of hospital strains in the department.

Conclusion. The study of the genetic structure of *A. baumannii* strains using PCR analysis of *OXA-23*, *ISAbal*, *csuE*, *tonB* genes enables to assess efficiently the epidemic relationship between strains, reveal a predominant genotype and identify epidemiologically important properties of the strains.

Key words: *Acinetobacter baumannii*; hospital strains; antibiotic resistance; biofilms; antibiotics.

In recent years non-fermentative gram-negative bacteria occur more often among the infectious agents causing complications in patients including those in burns units. A constantly increasing incidence of infections caused by *Acinetobacter spp.*, mostly *A. baumannii* has been registered [1–3].

Acinetobacter spp. are free living non-fermenting bacteria able to persist and multiply in a hospital setting using nearly all natural organic compounds as a substrate. They contaminate various solutions including some disinfectants (Furacilin, Rivanol, etc.), as well as medical instruments and equipment, especially in places with accumulated fluid. The main features of *Acinetobacter spp.* are resistance to many groups of antibacterial chemotherapeutic agents, the ability to form biofilms (both on the tissues of a living organism and polymeric materials used in medicine), the presence of the “quorum-sensing” signal system that enhances the protection of the bacteria from antibiotics,

disinfectants and the human immune system. The main virulence factors are a polysaccharide capsule, pili, an enzyme that breaks down the lipids of host tissues, a toxin that causes the death of white blood cells [1, 4, 5].

The microbiological monitoring of the formation of antibiotic resistance is an important method for assessing the effectiveness of antibiotic therapy and possibility of empirical administration of antibacterial chemotherapy drugs [6]. *A. baumannii* resistance to carbapenems is increasingly spreading in hospitals throughout the world. The major mechanism of resistance to this class of antibiotics is the effect of carbapenemases and oxacillinases. *Acinetobacters* have several classes of oxacillinases, but, according to some studies [7–9], the most important one is *OXA-23* and it is found most frequently in multiresistant strains. The *ISAbal* insertion element comprises promoters, which play an important role in the expression of genes encoding *OXA-23* that enhances antibiotic resistance

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in hospital environments. This insertion element can be considered as an epidemiologically significant marker of drug-resistant hospital strains of *A. baumannii*.

The protein encoded by the gene of the *tonB*-dependent receptor is part of the outer membrane of bacterial cells, responsible for iron absorption and it is a major virulence factor necessary for the survival of bacteria in the blood and lungs. The clones having the gene can cause bacteremia in patients over a short period of time [10]. *CsuE* is one of the genes involved in the regulation of biofilm formation. It is responsible for the presence of pili in bacterial cells [11]. *A. baumannii* strains are identified by the polymerase chain reaction (PCR) or multiplex PCR with *trpE*, *adk*, *mutY*, *ppa* constitutive genes, which are always available, as required for the basic processes in bacterial cell activity [12].

Burns units are characterized by a higher incidence of hospital *Acinetobacter* strains and their prolonged circulation in the hospital [13]. The results of the studies conducted in European countries show occurrence and rapid spread of "epidemic" *A. baumannii* clones with high affinity, i.e. hospital strains [14]. There is lack of publications of native authors on this subject, only data for some regions of the European part of Russia are available [15], similar studies have not been conducted in the Far East region.

Effective epidemiological surveillance requires identification of the causative agent for the strains and tracking of the genetic structure of the hospital population, which is fully possible only when with the use of molecular genetic techniques. In the present study we analyzed the strains obtained in the burns unit of the Far Eastern District Medical Center, Federal Medical and Biological Agency of Russia.

Currently, the world gold standard for genotypic analysis is MLST (multilocus sequence typing). Two schemes are used for *Acinetobacter*s: those of the Pasteur Institute (Pasteur's MLST) [16] and Oxford (PubMLST) [17]. Despite the obvious advantages of this technique, such as high resolution and standardization of conditions, MLST is available to a very limited number of laboratories due to the high cost of equipment and supplies. The proposed PCR method is much more affordable.

The aim of the investigation was to assess the nature of the spread of epidemiologically relevant *Acinetobacter baumannii* strains, to identify the predominant genotype, to determine the availability of hospital strains with the help of molecular genetic techniques.

Materials and Methods. We investigated 60 *A. baumannii* strains isolated in patients of the burns unit at the Far Eastern District Medical Center, Federal Medical and Biological Agency

of Russia in 2011–2013. The microorganisms were isolated and identified by microbiological techniques [18] and with the use of molecular genetic analysis [19]. DNA was isolated by boiling: a loop of the overnight culture was transferred into a 1.5 ml Eppendorf tube with 100 µl water, purified from nucleases, boiled for 5 min in a water bath, then centrifuged at 12,000 rev/min for 3 min. The supernatant was transferred to sterile tubes with an automatic micropipet and used for analysis.

The polymerase chain reaction was performed on a 1000TM ThermalCycler C (Bio-Rad, USA). The primers used are shown in Table 1.

The working mixture contained 1 unit of Taq DNA-polymerases (SibEnzyme, Russia), 1×buffer (10×: 600 mmol of Tris-HCl; 250 mmol of KCl; 15 mmol of MgCl₂; 100 mmol of 2-mercaptoethanol; 1% Triton X-100, pH=8.5), 0.8 mmol of deoxynucleoside triphosphate mixture (0.2 mmol each), 0.25 µmol of each primer and 50 ng of DNA.

OXA-23 gene amplification was performed under the following conditions: pre-denaturation at 94°C — 5 min; the subsequent 35 cycles at 94°C — 30 s, at 52°C — 40 s, at 72°C — 50 s and final chain completion at 72°C — 5 min. The other genes amplification was performed under the following conditions: pre-denaturation at 95°C — 5 min, 8 cycles at 95°C — 30 s, the annealing temperature was increased by 0.9°C beginning with 48°C at each cycle, at 72°C — 20 s, the subsequent 36 cycles at 95°C — 15 s, at 56°C — 20 s, at 72°C — 20 s and final completion chains at 72°C — 5 min. The amplification products were analyzed by electrophoresis in 2.0% agarose gel (Agarose Biotechnology Grade, Amresco, USA) staining DNA with ethidium bromide. The molecular weight marker was a standard set of DNA fragments multiple of 100 base pairs of nucleotides (SibEnzyme, Russia). The TotalLab software v. 2.01 was used to determine the size of the amplified fragments.

Table 1

Primers used for genotyping *A. baumannii* isolates

Primer	Nucleotide sequence	References
OXA-23	F: 5'-ACTTGCTATGTGGTTGCTTCTC-3' R: 5'-TGCAAGCTCTTAAATATTCAGC-3'	[8]
ISAbal	F: 5'-CAGGAATGCAGAAGTTG-3' R: 5'-CGACGAATACTATGACAC-3'	[8]
csuE	F: 5'-TAGCGGGCTGATGGCAATT-3' R: 5'-ACCCAGGGCTCTCAAAGAAG-3'	[11]
tonB	F: 5'-GGACTGGTGATAAAGCACTAT-3' R: 5'-GCCGCATAGAGTTATCACATC-3'	[10]
adk	F: 5'-TCAACCTGACTGCGTGAATGGTTGT-3' R: 5'-TACGTTCTACGATTTCTTCATCAGGTACATC-3'	[12]
mutY	F: 5'-TCGTGCCCGCAATTTGCATAAAGC-3' R: 5'-TAATGCCGGGTAGTGAATCCATTCTCTAG-3'	[12]
ppa	F: 5'-TGTTATGTACCAAATACTTTGTCTGAAGATGG-3' R: 5'-TGACGGCATCGATACCACCGTC-3'	[12]
trpE	F: 5'-TGAGATTGCTGAACATTTAATGCTGATTGA-3' R: 5'-TTGTACATTTGAAACAATATGCATGACATGTGAAT-3'	[12]

The presence or absence of amplicons was considered as a binary feature according to which a binary matrix was developed for all the studied *A. baumannii* isolates and primer sequences used. Later, each isolate was typed for all pairs of primers which resulted in obtaining combined genotypes. The genotype polymorphism was evaluated by a χ -squares test [20] for each pair of isolates using the ARLEQUIN v. 3.5 software [21]. The optimal dendrogram of MP (maximum parsimony tree) was searched by the PENNY algorithm [22] using PHYLIP (Phylogeny Inference Package) v. 3.67 software package [23]. Clusterization stability was assessed in 1,000 iterations of the bootstrap analysis [24]. The graphical image of the dendrogram was obtained using the TreeView software (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>).

Table 2

A. baumannii isolate genotyping by PCR analysis

Genotype	OXA-23	ISAbal	csuE*				tonB	Occurrence (%)
			a	b	c	d		
A	+	+	-	-	-	-	+	50
B	+	+	+	-	-	-	+	5
C	-	+	-	-	-	-	+	5
D	-	-	-	+	-	-	+	5
E	-	-	-	+	+	-	-	5
F	-	-	-	-	-	-	-	10
H	-	+	-	+	-	-	+	5
I	+	+	-	+	-	-	+	10
K	+	+	-	-	-	+	+	5

* a size of an amplified *csuE* gene fragment: 180 bp (a), 200 bp (b), 220 bp (c), 285 bp (d). Here: bp — base pairs of nucleotides.

Results and Discussion. The *adk*, *mutY*, *ppa* and *trpE* genes were amplified in all the studied *A. baumannii* strains. *OXA-23* and *ISAbal* genes were amplified in 70 and 80%, respectively, indicating the presence of resistance to carbapenems. The *csuE* gene was identified in 40% of the strains. They have four different modifications with different molecular weight. There are reasons to believe that it is caused by mutations in the gene, which indicate that the bacteria are predisposed to the formation of bacterial biofilms. This is typical of many hospital strains. The *tonB* gene, encoding the bacteria properties allowing bacteraemia in a short time is found in 15% of the strains. During the analysis, all the data on presence/absence of the feature for each strain were pooled, thus obtaining a combined genotype corresponding to a specific set of genes and a genetic characteristic of the strain. 9 combined genotypes were identified on the studied genes encoding antibiotic resistance and virulence factors. The most common genotype was A (50% of cases). F and I genotypes occurred in 10%, and the rest ones in 5% of cases (Table 2).

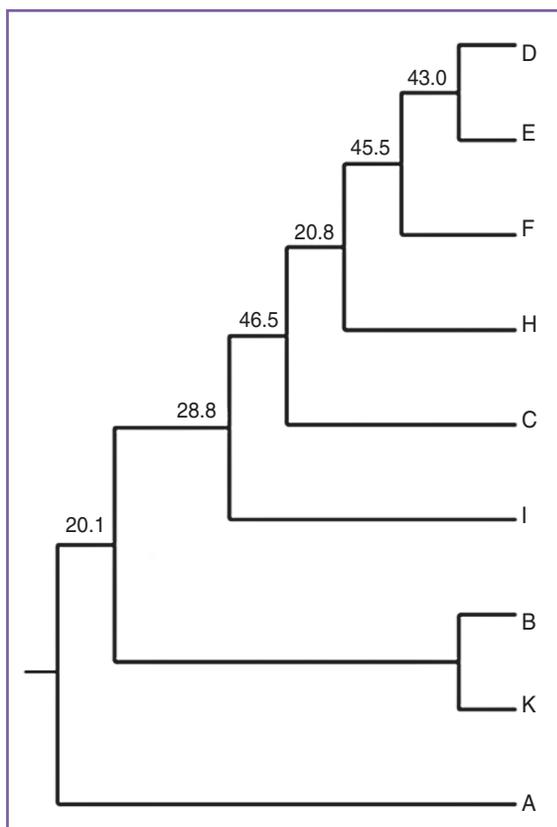
Assessment of genetic differences in *A. baumannii* genotypes, obtained by χ -test squares for each pair of isolates suggested that only I and F genotypes are significantly different at the 95% significance level (Table 3). No significant differences between the other genotypes were found. The MP-based dendrogram was drawn on the base of the analysis of the genetic polymorphism of the *A. baumannii* isolates (See the Figure).

Table 3

Genetic differentiation of A. baumannii isolates

Genotype	A	B	C	D	E	F	H	I	K
A		-	-	-	-	+	-	+	-
B	0.09066± 0.0017		-	-	-	-	-	-	-
C	0.08981± 0.0016	1.00000± 0.0000		-	-	-	-	-	-
D	0.09199± 0.0011	1.00000± 0.0000	1.00000± 0.0000		-	-	-	-	-
E	0.09165± 0.0015	1.00000± 0.0000	1.00000± 0.0000	1.00000± 0.0000		-	-	-	-
F	0.01574± 0.0006	0.33194± 0.0026	0.33358± 0.0019	0.33724± 0.0020	0.33191± 0.0015		-	-	-
H	0.09408± 0.0022	1.00000± 0.0000	1.00000± 0.0000	1.00000± 0.0000	1.00000± 0.0000	0.33340± 0.0023		-	-
I	0.01499± 0.0006	0.33328± 0.0028	0.32977± 0.0023	0.33343± 0.0027	0.33206± 0.0024	0.33073± 0.0026	0.33406± 0.0018		-
K	0.09075± 0.0007	1.00000± 0.0000	1.00000± 0.0000	1.00000± 0.0000	1.00000± 0.0000	0.33578± 0.0021	1.00000± 0.0000	0.33345± 0.0018	

Note. The scores of genetic differences are below the diagonal, above the diagonal — the significance of differences for p=0.05 significance level; “-”: insignificant difference, “+”: significant difference.



MP dendrogram drawn on the base of the analysis of gene polymorphism of *OXA-23*, *ISAbal1*, *csuE*, *tonB* genes of *A. baumannii* strains. Numbers indicate the estimated bootstrap support (percentage of 1,000 replicas)

The dendrogram as well as the data on the genetic differentiation of the studied isolates (See Table 3) indicate their close genetic affinity, as evidenced by the low values of the bootstrap support that does not exceed 50%.

Thus, the investigation of the genetic structure of 60 *A. baumannii* isolates by PCR analysis of *OXA-23*, *ISAbal1*, *csuE*, *tonB* genes indicate an insignificant level of genetic differentiation. The obtained data point to the persistent existence of hospital strains in the hospital environment and high incidence of nosocomial exogenous infection in patients as endogenous infection with different strains would provide a much greater variety of features. These data suggest the need to comply with all the requirements of infection control in hospitals and the use of the principles of rational antibiotic therapy in everyday medical practice, as well as the improvement and unification of surveillance techniques over the formation of hospital strains.

Compared with the MLST technique the proposed technique of using PCR to determine the spectrum of hospital strains is less costly, because the equipment used for this technique is available in most research and clinical laboratories, and the cost of supplies is significantly lower than that of the reagents used for sequencing. The results obtained by this technique

are easily reproducible and statistically significant, suggesting the possibility of its wide use.

Conclusion. The study of the genetic structure of *A. baumannii* strains with the help of PCR analysis of *OXA-23*, *ISAbal1*, *csuE*, *tonB* genes allows to assess effectively the presence of epidemic link between strains, identify the prevalent genotype and determine epidemically significant features of the strains.

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