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IMPROVEMENT OF *BACILLUS ANTHRACIS* LABORATORY DIAGNOSTIC METHODS IN AZERBAIJAN 2005-2016

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Objective: development of the method Bac. anthracis, SNP-genotyping using mini sequencing.

Material and methods: DNA of Bac. anthracisstrains, using reagents for PCR, methods for PCR-amplification, DNA sequencing and computer hardware, BLASTn on line resource and GenBank data base were used.

Results: Results by methods of real time allele-specific PCR amplification and direct sequencing were developed to show global phylogeographic patterns of Bac. anthracis strains to certain geographical areas. These genotypes were inherent Bac. anthracis genotypes, common in the territory of Georgia and Turkey.

Conclusion: method for the SNP-genotyping using the mini sequencing.

Keywords: *Bacillus anthracis, genotyping, mini sequencing, DNA, RAPS, PCR-method, SNP-analysis*

Anthrax is widespread special zoonotic diseases infection and is high risk for health of people. The difficulty of struggle with this infection is wide availability of its natural source and possessors, not learning of antigen structure of genome and microbial cells sufficiently according to biological characteristics of its excitant. The ability of excitant of anthrax to stay in the environment for a long time and identification of pathogenic bacilli creates an opportunity to use of newer, sensible and specific means and methods. Anthrax is an endemic disease for Azerbaijan. Every year over the past 10 years, an average of 10 sporadic cases per year are recorded, including outbreaks. There are 1.245 contaminated foci in the Azerbaijan, which are distribution on the different territories with several of physical and geographical peculiarities (Figure 1) [1, 2].

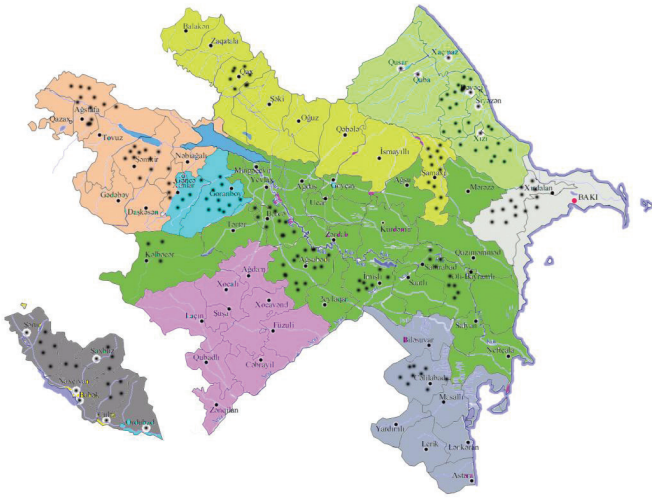

Figure 1. Distribution of anthrax foci and contaminated soil in Azerbaijan

Excitant of anthrax – *Bac. anthracis* is inactive gram-positive aerobic rod-shaped bacterium forming spore and capsule in the length of 6–10 mkm and in the width of 1–2 mkm. Forming of capsule is coded with 60 kDa plasmid. *Bac. anthracis* capsule consists of poli-g-D-glutamine and it may be appeared easily at the time of painting of drugs properly. *Bac. anthracis* produces three thermo labile proteins: edematous factor (EF-edematous factor), lethal factor (LF-lethal factor) and protective antigen (PA- protective antigen), which separately has no pathogen characteristics; toxic effect emerges only in combination with each other. *Bac. anthracis* strain will be virulent in the case that, when it produces both virulent (lethal and edematous) and possesses to the formation ability of capsule [3].

Periodic ignitions of anthrax and remaining of spore of *Bac. anthracis* on soil for a long time, also the opportunity to use of this microbe as biological arm in military and terror targets with high virulence makes its learning important. In this regard, improvement of only methods, diagnostics, prophylactics and treatment aids has not special importance. In addition, preparation of exact and effective methods of genetic typing of excitant is necessary for determination of the source of *Bac. anthracis* strains used at the time of ignition or at the time of terror act [4].

In Azerbaijan, the most commonly encountered anthrax is the cutaneous form. High sensible methods of genome analysis are sued widely for identification of excitants of bacterial diseases: polymerase chain reaction (PCR), sequencing (genotyping) and ribotyping.

Genotyping – is one of the most important analyses. It helps to forecast successful treatment chances, to determine the necessary doses of drugs and term of therapy. Different genotypes have different stability in treatment. It is very perspective direction of medicine. Genotyping– is a method based on the newest achievements in the area of biotechnology creating a condition to compare individually the genotypes of different strains. It creates a condition to prepare individual genetic passport of microorganisms by help of comparison of analysis and its deoxyribonucleic acid [5].

Laboratory diagnostics of all suspected human *Bac. anthracis* cases is carried out at the Republican Anti-Plague Station (RAPS) and the 4 regional anti-plague divisions. Until 2005, the diagnostics was performed only by bacteriological method. Antibiotic therapy which is administered to all suspected cases makes the bacteriological method of identification insufficient and problematic. In 2005, PCR was introduced in Azerbaijan to improve laboratory diagnostics method of detection of *Bac. anthracis* in human cases. Along with the new diagnostic method, also internationally and Azerbaijan Government's approved Biosafety methodology was introduced to the laboratories (Laboratory Biosafety Manual, 2004, WHO).The succession of stable and variable nucleotides of anthrax bacterium sections will give us an opportunity firstly to compare them with strains determined and discovered outside of the Republic. Method for the SNP-genotyping using the mini sequencing has been developed in Azerbaijan.

Excitant of anthrax – *Bac. anthracis* was considered genetic high monomorphic until recently. Individual differences of genomes of *Bac. anthracis* strains appeared after application of the latest methods of molecular typing basing on single nucleotide polymorphism areas of genome (SNP- Single Nucleotide Polymorphism). Genotyping of strains creates a condition to divide them to specific and genetic changed areas distinguished them for virulence. These researches will create a condition to identification of *Bac. anthracis* strains brought to the territory of Azerbaijan.

The goal of our researches is genotyping of anthrax strains recorded in the territory of Azerbaijan. Development of the method *Bac. anthracis*, SNP-genotyping using mini sequencing.

Material and methods. The materials used for investigated: materials from cattle, environmental samples, soil probes, materials from patients with cutaneous form. RAPS have been investigating anthrax used epidemiological and laboratory studies and surveillance have been conducted.Laboratory study includes isolation, identification, confirmation and genotyping.

Amplification was performed using Idaho Technology kit. Extracted samples tested by real-time PCR using the R.A.P.I.D and a Light cycler 2.0 instrument from Roche Diagnostics. The work was conducted in biological safety cabinet (BSC class IIA), using all required personal protective equipment (PPE), following guidelines and SOPs.

A special place among the analysis of genetic markers of strains of B. anthracis in the world practice, as well as other methods for molecular genotyping of anthrax's excitant occupy single nucleotide polymorphism [6].

A special place among the analysis of genetic markers of strains of *Bac. anthracis* occupied by SNP-method. The *Bac. anthracis* genotyping system, with an analysis of the 13 so-called "canonical" (canSNP), described by M. Van Ert.

On the basis of an analysis of a small number of markers, it is possible to accurately determine the belonging of a strain to one of the main groups of the molecular diversity of an anthrax microbe, to make a preliminary conclusion about its geographical origin. Currently, there is an on line database of such as MLVA bank for Microbes Genotyping-(Http://mlva.upsud.fr/mlvav4/genotyping/), with which you can compare the results. In this resource there is a section of SNP-typing of an anthrax microbe that allows you to correlate your data with those available in the database, classify them and conduct phylogenetic analysis of Tab1. When sequencing Azerbaijani strains, one of the sequencing methods was used-this is sequencing loci with SNP. The principle consists in the enzymatic completion of the primer, immediately behind the 3 'end of which follows a single nucleotide polymorphism in the complementary matrix, to one or more of the nucleotides, depending on the variant of the SNP allele. The results were detected by fluorescence labeling (fluorescence-labeled probes) [7].

Cultures grown at a temperature of 37 °C were used to isolate the DNA. The grown culture was taken from the cup by a loop and placed in epindorff, where 500 µl of NaCl and was added 500 µl of chloroform. The resulting suspension is vortexes for 1 hour every 15 minutes. Then this extinction is extracted. The sequence of operations of the method begins precisely with the isolation of DNA strains of *Bac. anthracis.* The samples were extracted using the Qiagen kit in accordance with the manufacturer's instructions. Then, PCR amplification of seven genome regions containing SNP followed with the isolated DNA template and the primers shown in table 1.

Table 1 – Primers for amplification of the genome with SNP

SNP	Nucleotide sequence of primers, 5'→3'	
	Forward	Reverse
A/B. Br.001	gaaggtctccaatttggatttaaaat	cgtgtgaacctttcggtaaatagtc
A.Br.002	aacgatacctaaaatcgataaag	ggcagaaggagcaagtaatgtt
A.Br.003	gctactgtcattgtataaaaacctccttt	cgcttgccaagctttttttc
A.Br.006	ccggaaattgctattagaacgaa	tcccaatctagcgtttttaagttca
A.Br.007	ttggtaacgagacgataaactgaataa	gccttggattggcgattg
A.Br.008	ttcgcaactacgctatacgttttagat	caaacggtgaaaaagttacaaatatacg
B.Br.003	catttattcgcatagaagcagatga	tgtgccatcaaataactctttctcaa

The reaction mixture was in the range of 10 µl containing: 5 µl contained 1x SYBR Green PCR master-mix (Applied Biosystem, Foster City, CA), two AS (allele-specific) forward primers, where the 3' ends correspond to one of the allele-states for the SNP locus. One of them forward primers: 0,15 µl Derived primer with concentration 10 µM and 0, 6 µl Ancestral primer with concentration 10 µM too. The reaction mixture also contained 0,15 µl Common Reverse primer with concentration 10 µM plus 3, 10 µl molecular grade H_2O and 1 µl of DNA template. DNA templates were produced from the Whole Genome Amplifications (WGA; Qiagen, Valencia, CA) or genomic DNA extracted by various types of preparations (standard heak soak or chloroform preparations). As a positive control used a reference Georgian strain. Genotyping were performed on an AppliedBiosystemCFx96 Touch Real-Time PCR Detection System. Thermo cycling parameters were: 95 ^{0}C for 5 min and then cycled at 95 ^{0}C for 30 s and 60 ^{0}C for 30 s, 72 ^{0}C – 40 s for 40 cycles. End-point PCR products were subject to melt analysis using a dissociation protocol comprising 95 ^{0}C for 30 s followed by incremental temperature ramping from 72 ^{0}C to 95 ^{0}C SYBR Green fluorescent intercity at 530 nm at each ramp interval and plotted against temperature.

Results. From 2005–2016 the presence of *Bac. anthracis* were tested by bacteriological and PCR methods. 252 swab samples suspected for *Bac. anthracis* were investigated in the RAPS and regional laboratories. Only Identified by all the laboratories *Bac. anthracis* cultures were submitted for confirmatory testing into the RAPS Museum of Pathogens, where they were further tested by PCR and bacteriological. Out of 252 swab samples 100 (50 %) were positive only by PCR. Only 13 (5 %) samples were positive by bacteriological and PCR method. The 139 samples were negative for *Bac. anthracis*, tested by both methods: bacteriological and PCR (Figure 2).

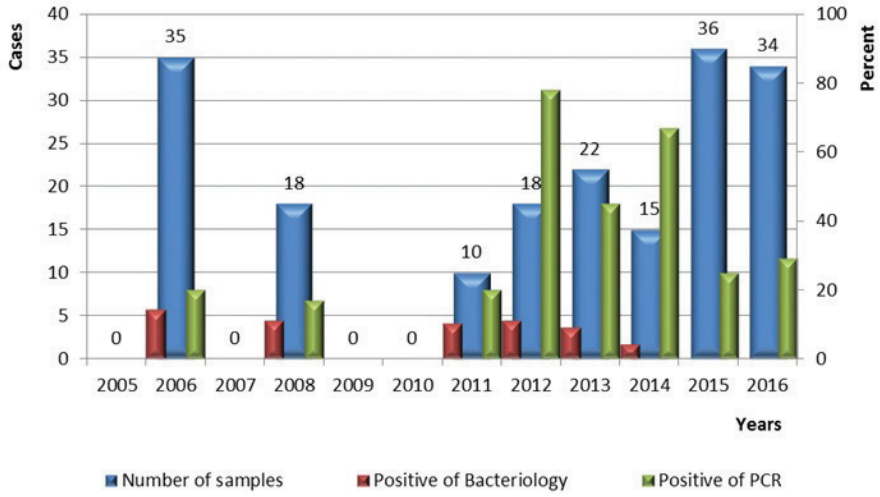

Figure 2. Number of cultures of *Bac. anthracis* in the Collection of Pathogens

In 2012, anthrax was observed in 14 events in Gakh region, Azerbaijan Republic. However, any molecular diagnostics of 14 events observed wasn't conducted.

As a result, was established the design of the detected single polymorphisms.

There are 28 strains of *Bac. anthracis* in the Collection of pathogens of the Republican Anti-Plague Station in Azerbaijan. 15 (53 %) of the strains are genotyped. Genotyping of *Bac. anthracis* strains through SNP analysis method.The amplification of alleles are illustrated for haploid template (*Bac. anthracis*) on the Figure 3 possessing a polymorphic SNP-state. Each amplification plot represents a single PCR reaction containing reverse "common" primer and two AS primers. Plots of the temperature-dissociation (melt) curve of the final PCR products are shown to the respective amplification plots (green arrows). AS PCR products are easily differentiated through temperature-dissociation (melt) curve analysis.

We investigated seven genetic populations of the pathogen *Bac. anthracis*. Assays are screened across ancestral and derived DNA. Polymorphic allele DNA templates (ancestral and derived, respectively), of equal DNA amounts and Nano Drop Tm measurements were screened on seven targeting *Bac. anthracis:* A. Br. 002, A. Br. 003, A. Br. 006, A. Br. 007, A. Br. 008, and B. Br. 003, A\B. Br. 001. (Figure 4).

These data allowed us to determine the Azerbaijan of ancestral root of *Bac. anthracis*, showing that its lies closer to a newly described "A" or "B" branches. If we discovered "ancestral state" forms, studies in that direction were stopped. In the presence of "derived state" forms we were continued further studies in the corresponding branch.In the process of genotyping of *Bac. anthracis* cultures using the SNP analysis method, is established that the researched Azerbaijani strains fall under the groups A/Br008 and A/Br003.These genotypes were inherent *Bac. anthracis* genotypes, common in the territory of Georgia and Turkey.

With the increased accessibility of whole genome sequencing, typing schemes based on single nucleotide polymorphisms (SNPs) provide both high resolution and highly accurate phylogenetic information although phylogenetic discovery bias must be taken into account. Results by methods of real time allele-specific PCR amplification and direct sequencing were developed togive the opportunity to place the Azerbaijani strains on the international phylogenetic tree (Figure5).

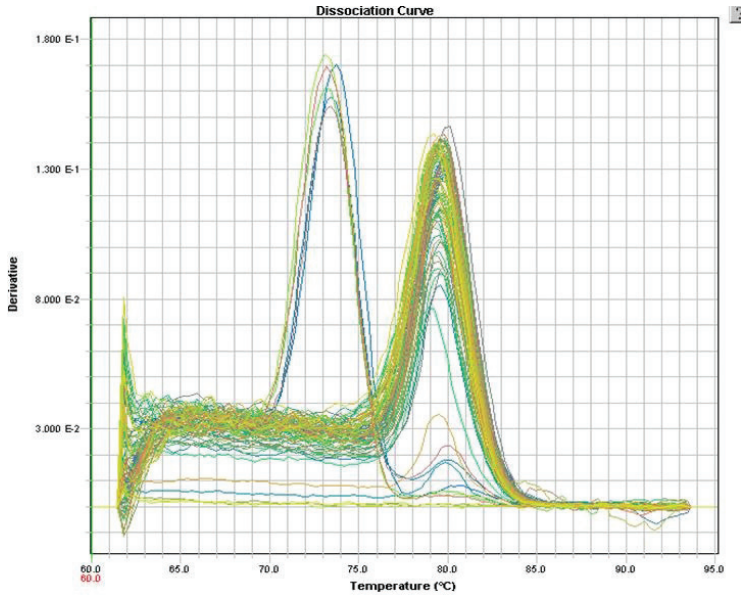

Figure 3, 4. SNP genotyping

Sample ID	Region	Years	A/B.Br001	B.Br.003	A.Br 006	A.Br.007	A.Br.008	A.Br.003	A.Br.002
1	Shabran	2001	DER	ANC	DER	ANC	DER	ANC	ANC
2	Shamkir	2002	DER	ANC	DER	ANC	DER	ANC	ANC
3	Shamkir	2003	DER	ANC	DER	ANC	DER	ANC	ANC
4	Shamkir	2003	DER (low)	ANC (low)	DER	ANC	DER (low)	**UND**	ANC
5	Shamkir	2006	DER	ANC	DER	ANC	DER	ANC	ANC
6	Shamkir	2006	DER	ANC	DER	ANC	ANC	**UND/ repeat**	ANC
7	Absheron	2006	DER	ANC	DER	ANC	DER	ANC	ANC
8	Shamkir	2008	DER	ANC	DER	ANC	ANC	DER	ANC
9	Gakh	2012	DER	ANC	DER	ANC	ANC	DER	ANC
10	Gusar	2013	DER	DER?	DER	ANC	DER (low)	ANC	ANC
11	Imishli	2013	DER	**DER/ ANC**	DER	ANC	DER (low)	ANC	ANC
12	Xachmaz	2014	DER	DER/ ANC	DER (low)	ANC	DER	ANC	ANC

The black tree at the top represents the phylogeny that would be obtained if all strains were completely sequenced and all SNPs sampled. In practice, a subset of reference strains is typically used to define a panel of SNPs.Only substitutions lying on the evolutionary pathway linking that pair will be detectable, as shownby the red and green trees. All secondary branches collapse to zero length, resulting in unusual linear phylogenies. Linear phylogenies derived from SNPs generated from different pairs of reference sequences can, however, be combined. In this case, most of the tree can be reconstituted. Only taxon B is left with a zero branch length, and an unknown history. While the secondary branches of the no reference strains remained invisible, the overlapping portions of the two linear phylogenies showed almost perfect correspondence in node placements. Such evolutionary study withfully viewed for the first time in Azerbaijanis particularly fascinating in *Bac. anthracis*, which is essentially frozen in evolutionary time between transmissions, while it sits and waits as a soil spore for its next chance to proliferate in a new host [7].

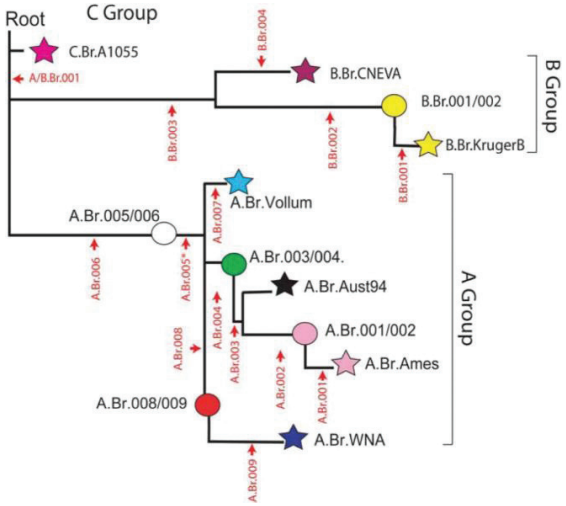

Figure 5. Placement of Azerbaijani strains of *Bac. Anthracis*

Conclusion. 1. This work forms a bridge between genomic data and important biological questions regarding the natural history, ecology, and tempo and mode of evolution of an important pathogen, *Bac. anthracis*. In the process of genotyping of *Bac. anthracis* cultures using the SNP analysis method, established that the researched Azerbaijani strains fall under the groups A/Br008 and A/Br003.

2. We report here the phylogeographic patterns of *Bac. anthracis* samples collected within the country of Azerbaijan. Phylogenic analysis of the researched strains was conducted, determining their place in the global hierarchy of anthrax germs.

3. SNP typing to characterize regional and global phylogeographic patterns of *Bac. anthracis.* Intensive regional sampling studies are not only invaluable for identify in endemic strains and for defining regional patterns of dissemination and cycling, but also build the foundation for understanding global patterns of spread. The results of genotyping will be used later in the identification of new strains of *Bac. anthracis*.

4. Determination of genotype of *B. anthracis* strains will facilitate the identification of territorial belonging and derivation of circulating and newly isolated strains of *Bac. anthracis* germ on the territory of Azerbaijan.

5. Located in a geographic on the Caspian Sea coast, and between Europe and Asia, our region has been impacted by ancient and modern human movement and trade. Such anthropogenic influences may have shaped the distribution of *Bac. anthracis* in our region and may prove to be important in understanding global phylogeographic patterns.

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СОВЕРШЕНСТВОВАНИЕ МЕТОДОВ ЛАБОРАТОРНОЙ ДИАГНОСТИКИ ПРИ РАБОТЕ С СИБИРСКОЙ ЯЗВОЙ В АЗЕРБАЙДЖАНЕ (2005–2016 ГГ.)

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Цель исследования. Разработка метода SNP-генотипирования Bac. anthracis с использованием минисеквенирования.

Материалы и методы. Исследовали ДНК штаммов Bac. anthracis с использованием реактивов для ПЦР, методов ПЦР-амплификации, ДНК-секвенирования, компьютерных программ on-line ресурса BLASTn и баз данных GenBank.

Результаты исследований. Данные, полученные в результате проведения метода аллель-специфической ПЦР-амплификации в режиме реального времени и прямого секвенирования показали определенную привязанность выделенных генотипов штаммов сибиреязвенного микроба к определенным географическим областям. Эти генотипы были присуще генотипам Bac. anthracis, распространенных на территории Грузии и Турции.

Выводы. Впервые в Азербайджане разработан метод SNP-генотипирования Bac. anthracis на основе минисеквенирования.

Ключевые слова: *сибирская язва, генотипирование, мини секвенирование, ДНК, ПЦР-метод, SNP-анализ*