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Research Article

High Resolution Genotyping of *Bacillus anthracis* Isolated from the Georgia- Azerbaijan Border Territory

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Abstract

B. anthracis is a member of a highly diverse group of endospore-forming bacteria. *B. anthracis* is considered one of the bacteria with a high degree of genetic homogeneity which makes it difficult to discriminate among the bacterial strains (Rume FI et al.).

Here we present study on genetic characteristics of environmental *B. anthracis* isolated from the Georgia-Azerbaijan border territory. We examined the genetic diversity of 62 *B. anthracis* isolates from Georgia-Azerbaijan border by *B. anthracis* MLVA-25 assay to better understand the dynamic of anthrax in this area. It was found these *B. anthracis* isolates were conserved. Elucidation of the molecular characteristics and relationships between Georgian and Azerbaijani *B. anthracis* strain populations will aid in the identification and tracking of strains and their origins.

Introduction

Anthrax is a bacterial zoonosis caused by *Bacillus anthracis*, a spore-forming, soil-borne bacterium with a remarkable ability to persist in the environment. Found on nearly every continent, the disease is considered a re-emerging zoonotic disease, and despite the development of anthrax vaccines for animals and humans, the disease continues to be endemic in many countries, including Georgia and Azerbaijan.

B. anthracis is a member of a highly diverse group of endospore-forming bacteria. The genus Bacillus contains at least 51 described species and many other species of uncertain taxonomic status. *B. anthracis* has been classified as a "Category A" organism by the US Centers for Disease Control and Prevention (CDC) and is considered a potential weapon for bioterrorism.

Animal transmission of *B. anthracis* is classically defined as ingestion of soils, contaminated plants, or contaminated water, as well as mechanical transmission by flies [1-3]. The toxins produced by the vegetative form of the bacterium are associated with virulence and differ from the toxins produced by other Bacillus species. Due to the environmental stability of spores, *B. anthracis* can remain viable in soil for many years and because its persistence does not necessarily depend on animal reservoirs [4], *B. anthracis* is extremely difficult to eradicate from endemic areas. Anthrax has recently re-emerged as a veterinary and human public health concern in Georgia. In addition to expanding geographically, the number of reported human cases (457) from 2010-2018 has increased nearly three-fold over the previous three years and more than 40-fold since 1985-1987 [5,6]. Moreover, approximately 2,000 *B. anthracis* foci are registered in Georgia, of which more than 20% are active. Recent research has shown new areas of emergence and clustering around urban centers in Georgia, which contrasts with the normal pattern of high infectivity associated with rural agriculture or remote areas [5,6]. In Azerbaijan, *B. anthracis* has been responsible for large outbreaks of anthrax in humans and livestock. Although reporting is more infrequent in Azerbaijan than in Georgia, anthrax has persisted for decades across a wide geographic expanse. The disease is considered to be sporadic in Azerbaijan although it is bordered by countries that are hyper-endemic [7], and recent evidence has shown that distribution of anthrax in Azerbaijan has undergone changes in its occurrence and spatial distribution [5]. Given the recent re-emergence of the disease in Georgia, there is growing concern over the status of the disease in Azerbaijan. Recent human outbreaks have raised concerns over the re-emergence of the disease, especially given the relatively few reported livestock cases in relation to these human cases. Although the region is experiencing an increased number of anthrax cases, few scientific efforts have been made to link trans-boundary outbreaks of anthrax using molecular characterization and geographic modeling.

In general, the geographic distribution of *B. anthracis* has been shown to be constrained by a combination of environmental factors such as temperature and precipitation [8,9]. While soil pH of 6.0 or above (alkaline) and factors such as rich organic content and high calcium levels are generally considered to be suitable conditions for the persistence of *B. anthracis* [4]. Research has also suggested that specific ecological affinities may contribute to the geographic variation in the distribution of genotypes [9].

Elucidation of the molecular characteristics and relationships between Georgian and Azerbaijani *B. anthracis* strain populations will aid in the identification and tracking of strains and their origins. In addition, genetic data may provide a mechanism for a retrospective epidemiological trace-back and for studying transmission dynamics, both of which will contribute to in-depth knowledge of the distribution and ecology of *B. anthracis* in Georgia, Azerbaijan, and globally.

Here we present study on genetic characteristics of environmental *B. anthracis* isolated from the Georgia-Azerbaijan border territory.

Biosafety and biosecurity overview

All of the studies presented in this paper were adhered to the biosafety recommendations contained in the 5th edition of the

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Biosafety in Microbiological and Biomedical Laboratories (BMBL) Manual published jointly by the CDC and NIH (relevant text from the BMBL Manual is reproduced below for each pathogen).

Safety issues

Safety at sample collection sites was ensured by the use of standard PPE, including: Tyvek coveralls, boots (12 inches high), N95 respirators, safety goggles, disposable hats, and double gloves (inside pair taped to the sleeves of the coverall). Staff at the collection sites have been using this PPE for many years, and are skilled in the safe handling of potentially infectious material.

Materials and Methods

Bacillus anthracis strain culture and inactivation

In this study, we analyzed 62 *B. anthracis* strains isolated from soil samples collected in the Georgia-Azerbaijan border territory. *Bacillus anthracis* isolates from pure cultures were grown on 5% Sheep Blood Agar (SBA) plates (Eliava Media Production, Georgia) at 37°C for 24 hours. Several loops of culture were transferred to 1.5mL micro centrifuge tubes and heat-inactivated in an autoclave at 121°C for 20 minutes [13].

DNA isolation and sterility testing

Sterile genomic DNA was extracted by using QIAamp DNA Mini Kits (Qiagen, USA) according to the manufacturer's instructions.

The sterility of samples was checked by pipetting 5% of the final volume of the DNA and incubating at 37°C in the same growth media used in bacterial culturing. To confirm sterility, at day 3 and day 7, 5µL of isolated DNA was placed on 5% SBA and incubated at 37°C. If no growth was observed after 72 hours at either time point, then the preparation was considered sterile. Primary and secondary containers were decontaminated with 1% sodium hypochlorite for 30 minutes, and stored at -20°C. After surface decontamination, sterile samples could be handled safely under biosafety level 1 or 2 containment.

Before genotyping, DNA quality and concentration of samples was measured using a NanoDrop 2000 instrument (Thermo Fisher Scientific, USA).

Genotyping using Multiple Locus Variable number tandem repeat Analysis

Genetic subtyping of *B. anthracis* using Multiple Locus Variable number tandem repeat Analysis (MLVA) was performed by a 25-marker MLVA genotyping scheme (MLVA-25) described previously [14] but adapted to a 5-dye Applied Biosystems platform (ABI 3130xl). The 25 PCR primer pairs were divided into four groups: Multiplex-A (eight loci including CG-3, Bams-44, Bams-3, vrrB-2, Bams-5, Bams-15, Bams-1 and vrrC-1), Multiplex-B (six loci including Bams-13, vrrB-1, Bams-28, vrrC-2, Bams-53 and Bams-31), Multiplex-C (five loci including Bams-25, vrrA, Bams-21, Bams-34 and Bams-24), and Multiplex-D (six loci including Bams-51, Bams-22, Bams-23 , Bams-30, pXO^{-1} and pXO^{-2}). Multiplex PCR was performed in a 15-µl reaction volume combining 1×PCR buffer, 0.2mM each of four deoxynucleoside triphosphates, 5mM $MgCl_2$, 1 U of Platinum Taq DNA polymerase with optimized concentrations of pre-mixed primers. The PCR conditions were as follows: initial denaturation at 95°C for 5 min, and then 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s, with a final extension of 72°C for 5 min and 4°C hold. After amplification, 2µL of each PCR reaction was diluted 100-fold in 198µL of molecular grade water. A denaturation solution/sizing standard solution was prepared from 18.7µL of Hi-Di formamide and 0.3µL of 1200 LIZ size standard; 19µL of the resulting solution and 1µL of the diluted multiplex samples were added to the wells of an ABI platform-compatible plate, e.g., MicroAmp Optical 96-well Reaction Plate (Life Technologies). Samples were denatured in a GeneAmp PCR System 9700 (Applied Biosystems) for five minutes at 95°C and then placed on ice for three to five minutes. PCR products for the 25 loci were resolved by capillary electrophoresis on an ABI Prism 3130xl automated fluorescent capillary DNA sequencer (Applied Biosystems). Amplicons were sized using ROX (carboxy-X-rhodamine)-labeled molecular ladder Liz 1200 (MapMaker 1000; Bioventures Inc., Murfreesboro, TN, USA) and Gene Mapper software version 4.0 (Applied Biosystems).

Data analysis

For each bacterial strain, amplicons from each of the 25 VNTR loci were normalized according to the expected fragment sizes across the diversity of *B. anthracis*. The higher expected fragment size was used if the difference between the actual fragment size and the expected fragment size was greater than half of the repeat length for that locus; otherwise the next lower expected fragment size was taken if the difference between the actual fragment size and the expected fragment size was less than half a repeat length. The resulting data were analyzed with Bionumerics software package version 7.6 (Applied-Maths, Saint-Martens-Latem, Belgium) as a character dataset. Clustering analysis was done using the categorical coefficient and Unweighted Paired Group Method Arithmetic Average (UPGMA) to generate similarity matrices and dendrograms from this MLVA dataset. Dendrograms are presented with percent similarity.

Calculation of discriminatory power. The discriminatory power of *B. anthracis* MLVA-25 was presented using the Hunter-Gaston diversity index. Diversity index (Hunter-Gaston) of each locus was calculated using Bionumerics software.

Results

To evaluate the discriminatory power of the selected loci, the Hunter and Gaston discrimination index was calculated for each of the 25 loci used in this study. Among these 62 isolates, the Diversity index value of each locus ranged from 0.46 to 1.00 (Figure 1). Among the 25 loci, there were ten loci for which the allelic diversity index was 1.00, while the allelic diversity index was lowest (0.46) for Bams 3.

We examined the genetic diversity of 62 *B. anthracis* isolates from Georgia-Azerbaijan border by *B. anthracis* MLVA-25 assay to better understand the dynamic of anthrax in this area. It was found these *B. anthracis* isolates were conserved. Their similarity was 91.6% (Figure 2). When a cutoff value of 93% similarity was applied to define the MLVA cluster, there were two MLVA strain clusters (genotypes) in this collection of 62 isolates. Cluster A is represented by 33 isolates while the cluster B contains 29 isolates. Each cluster has several sub-clusters. Cluster A strains came from three regions (Gardabani, Lagodekhi and Marneuli) while cluster B came from seven regions (Gardabani, Lagodekhi, Rustavi, Sagarejo, Sighnaghi, Axmeta and Dedofliwyaro). Two regions (Gardabani and Lagodekhi) were represented by both cluster A and B. Marneuli only had cluster

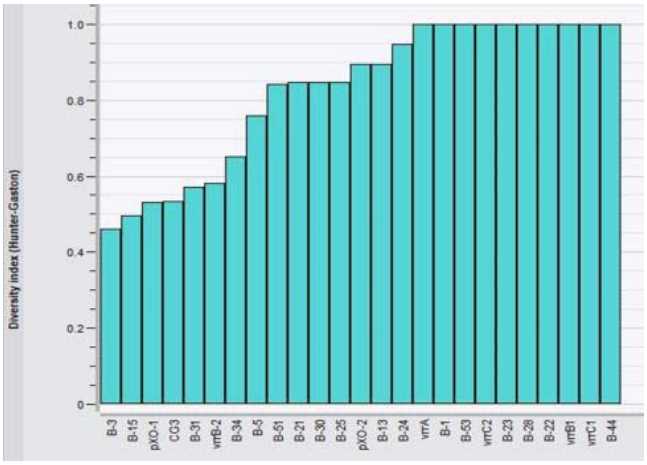


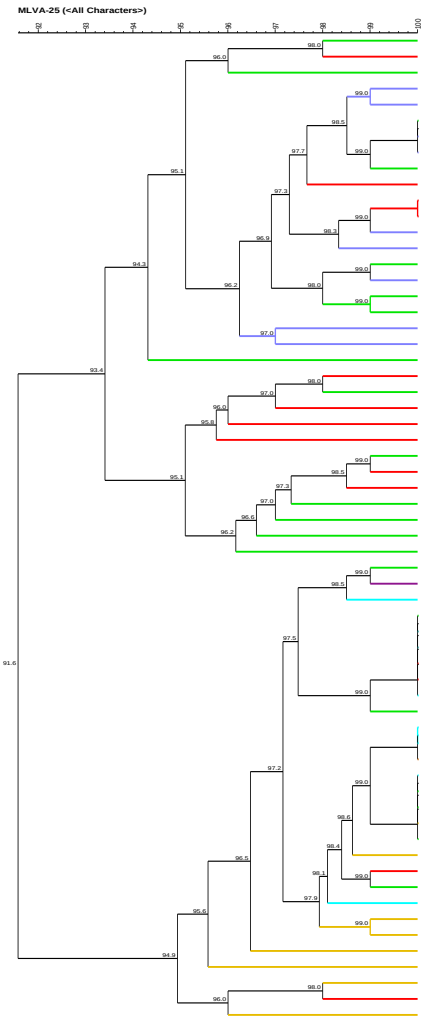
Figure 1: The diversity index value of each locus in 62 isolates from Georgia-Azerbaijan border.

A strains. Five regions (Rustavi, Sagarejo, Sighnaghi, Axmeta and Dedofliwyaro) only yielded cluster B isolates in this sampling.

Discussion

B. anthracis is considered one of the bacteria with a high degree of genetic homogeneity which makes it difficult to discriminate among the bacterial strains [15]. The high genetic homogeneity is most caused by its spore survival capacity which has allowed *B. anthracis* to multiply a relatively limited number of times during its evolution. MLVA is a standard assay for bacterial genotyping and has proved to be useful for molecular typing for *B. anthracis* [16]. There are different MLVA assays used to characterize and differentiate *B. anthracis* strains on different instrument platforms [17,18,14]. To obtain an increased power of discrimination, a 25-locus MLVA assay was applied to characterize 62 strains of *B. anthracis* collected in the Georgia-Azerbaijan border region. Of these 25 loci tested, ten loci had a diversity index of 1.00 to provide enough discriminatory power to differentiate *B. anthracis* isolates to strain level.

According to the results of the MLVA-25 genotyping done in this study, there were two MLVA strain types (genotypes) in this collection of 62 isolates. Looking at geographic distribution, three regions (Gardabani, Lagodekhi and Marneuli) had strains from cluster A while seven regions (Gardabani, Lagodekhi, Rustavi, Sagarejo,



Gardabani	GG-27-8	NCDC	Kvemo-Kartli	Gardabani	2016
Lagodekhi	GG-27-11	NCDC	Kakheti	Lagodekhi	2016
Gardabani	GG-27-7	NCDC	Kvemo-Kartli	Gardabani	2016
Marneuli	GG-27-30	NCDC	Kvemo-Kartli	Marneuli	2016
Marneuli	GG-27-31	NCDC	Kvemo-Kartli	Marneuli	2016
Gardabani	GG-27-23	NCDC	Kvemo-Kartli	Gardabani	2016
Marneuli	GG-27-25	NCDC	Kvemo-Kartli	Marneuli	2016
Marneuli	GG-27-32	NCDC	Kvemo-Kartli	Marneuli	2016
Gardabani	GG-27-22	NCDC	Kvemo-Kartli	Gardabani	2016
Lagodekhi	GG-27-21	NCDC	Kakheti	Lagodekhi	2016
Lagodekhi	GG-27-16	LMA	Kakheti	Lagodekhi	2016
Lagodekhi	GG-27-20	NCDC	Kakheti	Lagodekhi	2016
Marneuli	GG-27-27	NCDC	Kvemo-Kartli	Marneuli	2016
Marneuli	GG-27-28	NCDC	Kvemo-Kartli	Marneuli	2016
Gardabani	GG-27-24	NCDC	Kvemo-Kartli	Gardabani	2016
Marneuli	GG-27-33	NCDC	Kvemo-Kartli	Marneuli	2016
Gardabani	GG-27-18	NCDC	Kvemo-Kartli	Gardabani	2016
Gardabani	GG-27-19	NCDC	Kvemo-Kartli	Gardabani	2016
Marneuli	GG-27-26	NCDC	Kvemo-Kartli	Marneuli	2016
Marneuli	GG-27-29	NCDC	Kvemo-Kartli	Marneuli	2016
Gardabani	GG-27-1	NCDC	Kvemo-Kartli	Gardabani	2016
Lagodekhi	GG-27-13	NCDC	Kakheti	Lagodekhi	2016
Gardabani	GG-27-17	NCDC	Kvemo-Kartli	Gardabani	2016
Lagodekhi	GG-27-14	NCDC	Kakheti	Lagodekhi	2016
Lagodekhi	GG-27-10	NCDC	Kakheti	Lagodekhi	2016
Lagodekhi	GG-27-15	LMA	Kakheti	Lagodekhi	2016
Gardabani	GG-27-5	NCDC	Kvemo-Kartli	Gardabani	2016
Lagodekhi	GG-27-9	NCDC	Kakheti	Lagodekhi	2016
Lagodekhi	GG-27-12	NCDC	Kakheti	Lagodekhi	2016
Gardabani	GG-27-6	NCDC	Kvemo-Kartli	Gardabani	2016
Gardabani	GG-27-3	NCDC	Kvemo-Kartli	Gardabani	2016
Gardabani	GG-27-2	NCDC	Kvemo-Kartli	Gardabani	2016
Gardabani	GG-27-4	NCDC	Kvemo-Kartli	Gardabani	2016
Gardabani	GG-27-38	NCDC	Kvemo-Kartli	Gardabani	2016
Rustavi	GG-27-115	LMA	Kvemo-Kartli	Rustavi	2017
Sagarejo	GG-27-47	NCDC	Kakheti	Sagarejo	2016
Gardabani	GG-27-43	NCDC	Kvemo-Kartli	Gardabani	2016
Sighnaghi	GG-27-45	NCDC	Kakheti	Sighnaghi	2016
Sighnaghi	GG-27-58	NCDC	Kakheti	Sighnaghi	2016
Lagodekhi	GG-27-61	NCDC	Kakheti	Lagodekhi	2016
Lagodekhi	GG-27-72	NCDC	Kakheti	Lagodekhi	2017
Sagarejo	GG-27-73	NCDC	Kakheti	Sagarejo	2017
Gardabani	GG-27-34	NCDC	Kvemo-Kartli	Gardabani	2016
Sagarejo	GG-27-74	NCDC	Kakheti	Sagarejo	2017
Sagarejo	GG-27-76	NCDC	Kakheti	Sagarejo	2017
Axmeta	GG-27-84	LMA	Kakheti	Axmeta	2017
Sagarejo	GG-27-66	NCDC	Kakheti	Sagarejo	2017
Gardabani	GG-27-67	LMA	Kvemo-Kartli	Gardabani	2017
Gardabani	GG-27-68	LMA	Kvemo-Kartli	Gardabani	2017
Dedofliwyaro	GG-27-70	NCDC	Kakheti	Dedofliwyaro	2017
Gardabani	GG-27-81	LMA	Kvemo-Kartli	Gardabani	2017
Dedofliwyaro	GG-27-108	NCDC	Kakheti	Dedofliwyaro	2017
Lagodekhi	GG-27-64	NCDC	Kakheti	Lagodekhi	2016
Gardabani	GG-27-80	LMA	Kvemo-Kartli	Gardabani	2017
Sagarejo	GG-27-89	NCDC	Kakheti	Sagarejo	2017
Dedofliwyaro	GG-27-69	NCDC	Kakheti	Dedofliwyaro	2017
Dedofliwyaro	GG-27-79	LMA	Kakheti	Dedofliwyaro	2017
Dedofliwyaro	GG-27-97	NCDC	Kakheti	Dedofliwyaro	2017
Dedofliwyaro	GG-27-107	NCDC	Kakheti	Dedofliwyaro	2017
Dedofliwyaro	GG-27-101	LMA	Kakheti	Dedofliwyaro	2017
Lagodekhi	GG-27-116	LMA	Kakheti	Lagodekhi	2017
Dedofliwyaro	GG-27-119	LMA	Kakheti	Dedofliwyaro	2017

Figure 2: Dendrogram based on MLVA-25 of *B. anthracis* isolates.

Sighnaghi, Axmeta and Dedofliwyaro) had strains from cluster B. Only two regions (Gardabani and Lagodekhi) have both cluster A and B. This distribution suggests that *B. anthracis* has circulated the most in restricted area. Their rate of spread is relatively slow. It might be due to diversity of environmental conditions and economic activity in these territories [19]. Both Georgia and Azerbaijan have reported sporadic and focal outbreaks of *B. anthracis* cases in animals and human beings [20-23]. Thus active surveillance is important to prevent and control *B. anthracis* outbreaks. Based on our knowledge, this is first report of high resolution genetic characterization of *B. anthracis* circulating in the Georgian-Azerbaijan border region. This provides an initial snapshot of the distribution of *B. anthracis* genetic subtypes in this area, as well as an important tool for active surveillance to monitor the *B. anthracis* circulated in the Georgian-Azerbaijan border region in the future [24-29].

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