Part 1. Veterinary medicine

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PHYLOGENETIC ANALYSIS OF UKRAINIAN *BACILLUS ANTHRACIS* STRAINS

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Summary. In many countries anthrax is a common zoonotic disease which poses a serious threat to human and animal health. Sporadic cases of anthrax occur each year both among farm animals and humans in Ukraine. The cutaneous form of anthrax is the most widespread in Ukraine. The capability of *Bacillus anthracis* spores to remain viable in soil for decades, as well as the possibility to use this pathogen as biological terror agent make effective diagnostic and research capabilities extremely important. This comprises molecular methods including state-of-the-art methods for accurate genotyping of *B. anthracis* strains. A total of 12 *B. anthracis* DNA samples from a Ukrainian strain collection were studied by qPCR to confirm chromosomal and plasmid markers. To characterize regional and global phylogeographic relationships of these strains, canonical Single Nucleotide Polymorphism analysis (canSNP) and Multiple-Locus Variable-number of tandem repeat Analysis (MLVA-25) were conducted. *B. anthracis* chromosomal DNA-markers (dhp61 and gyrA) as well as those of the pXO1 plasmid could be detected in all 12 DNA samples. However, only 5 out of 12 tested strains contained the pXO2 plasmid-marker. All pXO2 positive strains group into the A.Br.008/009 SNP-clade, which belongs to the major 'A' branch of *B. anthracis*. MLVA-25 analysis suggested that Ukrainian *B. anthracis* genotypes are related to strains from Southern Europe (in particular, to Bulgarian, Greek, and Italian isolates). In contrast, the pXO2- negative strains might be related to the Russian vaccine strain STI as they grouped to A.Br.008/011 canSNP group. The infrequent occurrence of anthrax in the country of Ukraine is likely caused by a heterogeneous population of *B. anthracis*. This population is phylogenetically composed of at least two different canSNP groups of the world-wide dominating A-branch of the pathogen. While one group might stem from environmental recovery of live vaccine strains used in Ukraine (or the former Soviet Union in the past) the other one, the A.Br.008/009 group, could be enzootic as indicated by the presence of related strains in countries of southeastern Europe in relatively close geographical vicinity to Ukraine.

Keywords: anthrax, genotyping, PCR, HRM, canonical SNP, MLVA, Ukraine

Introduction. Anthrax, caused by bacterium *Bacillus anthracis,* is a zoonotic disease with a natural transmission cycle involving wildlife, livestock and humans (Van Ert et al., 2007). Infection with the disease poses a serious threat to human and animal health due to its mortality especially for animals (Purcell, Worsham and Freidlander, 2007). In addition, world-wide vigilance for anthrax is high due to the agent's potential to be used for nefarious purposes including bioterrorism which provides evidenced by the letter attacks in the United States in 2001 (Hoffmaster et al., 2002). In addition, anthrax was developed as potential biological weapon by several countries in the past, including the United States, the United Kingdom and the former Soviet Union (Keim et al., 2004). The ability of *B. anthracis* spores to remain viable in soils for decades simplifies both their isolation from the environment and dissemination as a biological weapon (Martin, Christopher and Eitzen, 2007). Importantly, the capability to form dormant and highly persistent spores in the environment plays major role in the ecology and evolution of the anthrax pathogen (Keim et al., 2000). Notably, during the spore phase, evolution rate is greatly reduced limiting the degree of genetic diversity found among isolates of this species (Van Ert et al., 2007). Therefore, not only reliable anthrax diagnosis is needed but also bio forensic capabilities including state-of-the-art methods for accurate genotyping of *B. anthracis* strains.

Two molecular approaches, namely Multiple-Locus Variable-number tandem repeat Analysis (MLVA) and whole genome Single Nucleotide Polymorphism (SNP) discovery and analysis, have greatly enhanced the identification of genetic markers for analysis of phylogenetic relationships among *B. anthracis* isolates (Keim et al., 2000; Pearson et al., 2004; Smith et al., 2000).

The *B. anthracis* global phylogeny is divided into three major lineages: A, B, and C. The A clade is by far the largest group and geographically the most wide-spread

genotype (causing 89.6% of all cases) (Van Ert et al., 2007). The A sub-linage radiates into multiple closely related and widely dispersed subgroups (Van Ert et al., 2007; Keim et al., 1997, 2000). The topological complexity among and within these groups, can be reliably resolved by using whole genome sequences to discover a relatively small number of SNPs (a few hundred). Once identified, these SNPs are highly discriminatory characters with high consistency in phylogenetic reconstructions (Pearson et al., 2004, 2009).

Ukraine is a large agrarian country in Eastern Europe. It has reported livestock outbreaks for more than a century (Gierczyński et al., 2004). At the turn of the 20th century, anthrax was a major animal and public health concern in the Russian Empire (which included a large part of Ukraine) (Korotich and Pogrebnyak, 1976), throughout the Soviet Period and since independence in 1991 (Bezymennyi et al., 2014). According to statistical data, 24,954 outbreaks of anthrax were detected in Ukraine throughout the period from 1920 to 2014 (Bobyliova et al., 2001; Bobyliova and Mukharska, 2002; Maly et al., 2013; data from OIE WAHIS). More than 4,500 anthrax burial sites are known in the Ukraine, and 60% of these are classified as old (grave sites from 1954 and older) (Zaviriuha, Yanenko and Zaviriuha, 2015). Moreover, sporadic infrequent cases of anthrax in Ukraine might be caused by naturally and anthropogenic re-release of spores from soil at old burial sites posing a constant risk of re-emergence of anthrax disease.

Currently, there is still a complete lack of information on the phylogenetic diversity of Ukrainian *B. anthracis* strains.

The aim of the study was genotyping of 12 *B. anthracis* DNA samples by canonical Single Nucleotide Polymorphisms analysis (canSNP) and Multiple-Locus Variable-number of tandem repeat Analysis (MLVA-25) for better phylogenetic placement of Ukrainian *B. anthracis* strains within the global diversity of the pathogen.

Materials and methods. A total of 12 *B. anthracis* DNA samples from Ukrainian strain collection of the State Scientific Control Institute of Biotechnology and Strains of Microorganisms (SSCIBSM, Kyiv, Ukraine) were isolated from live strains cultivated on soft agar and then re-inoculated on Hottinger broth. DNAs were prepared using 'Tissue DNA Isolation Kit' (MO BIO Laboratories, Inc., USA). Work was done at the State Scientific and Research Institute of Laboratory Diagnostics and Veterinary and Sanitary Expertise (SSRILDVSE, Kyiv, Ukraine).

DNAs were further analyzed at the Bundeswehr Institute of Microbiology (Munich, Germany) by qPCR to confirm the presence of anthrax chromosomal and plasmid markers. Metadata for the samples' origins is shown in Table 1 and Fig. 1.

To confirm the presence of specific DNA of *B. anthracis* chromosomal and plasmid markers, samples were analyzed by real-time qPCR using specific primers and probes for *dhp61* (chromosome), *pagA* (plasmid pXO1) and *capC* (plasmid pXO2) (Antwerpen et al., 2008; Rume et al., 2016). Canonical Single Nucleotide Polymorphism analysis (canSNP) was conducted as published (Derzelle et al., 2011; Derzelle, 2015). Primers and working concentrations are summarized in Table 2.

For VNTR typing of the DNA samples, we employed the MLVA-25 scheme as previously described by Lista et al. (2006). This collection of 25 loci represents all VNTR loci in the MLVA-8, MLVA-15 schemes (Keim et al., 2000; Beyer et al., 2012; Thierry et al., 2014; Leski et al., 2009) (Table 3).

From the MLVA data a UMPGA-tree was built using BioNumerics software (version 6.6).

Figure 1. Map of Ukraine with locations where studied samples originate from: 1 — BA-C-10-Cher (Khotyn district, Chernivtsi region); 2 — BA-D-12-Mel (Voznesenka village, Melitopol district, Zaporizhia region, Ukraine); 3 — BA-C-12-Sm (Makiivka village, Smila district, Cherkasy region); 4 — State Scientific Control Institute of Biotechnology and Strains of Microorganisms

Table 1 — Metadata of *B. anthracis* DNA samples from the SSCIBSM Strain Depositary

#	ID	Originating institution	Country	Source	Year
1	BA-C-10-Cher	SSRILDVSE	Khotyn district, Chernivtsi region, Ukraine	cattle	2010
2	BA-D-12-Mel		Voznesenka village, Melitopol district, Zaporizhia region, Ukraine		2012
3	BA-C-12-Sm		Makiivka village, Smila district, Cherkasy region, Ukraine		2012
4	55 VNIIVViM	Federal Research Center of Virology and Microbiology	Russia	vaccine live stock	2007
5	M-71	SSCIBSM	Ukraine		2001
6	K-79-Z	Institute of Veterinary Medicine of the NAAS			1997
7	B. anthr. SB	SSCIBSM			1997
8	B. anthr. 55	missing data	Russia		2012
9	Tsenkovskii-II				1997
10	STI				2007
11	Sterne 34F2	SSCIBSM	Ukraine		2015
12	UA-07		Ukraine		2007

Table 2 — Sequences and concentration of primers used for canSNP assay

canSNP	Name	Forward primer (5′→3′)	Reverse primer (5′→3′)	Concentration in duplex, μM
A.Br.001	BA1A	GTGGTAAGGCAAGCGGAAC	ACGGTTTCCCTTTATCATCG	0.20
A.Br.002	BA2	GCAGAAGGAGCAAGTAATGTTATAGGT	CCTAAAATCGATAAAGCGACTGC	0.15
A.Br.003	BA3	AAAGGAATTTAGATTTTCGTGTCG	ATAAAAACCTCCTTTTTCTACCTCA	0.20
A.Br.004	BA4	ATCGCCGTCATACTTTGGAA	GGAATTGGTGGAGCTATGGA	0.15
A.Br.006	BA5	GCGTTTTTAAGTTCATCATACCC	ATGTTGTTGATCATTCCATCG	0.20
A.Br.007	BA6	TTACAAGGTGGTAGTATTCGAGCTG	TTGGTAACGAGACGATAAACTGAA	0.20
A.Br.008	BA7	CCAAACGGTGAAAAAGTTACAAA	GCAACTACGCTATACGTTTTAGATGG	0.20
A.Br.009	BA8	AATCGGCCACTGTTTTTGAAC	AGGTATATTAACTGCGGATGATGC	0.25

Table 3 — The list of 25 published *B. anthracis* VNTR markers

Locus	Forward primer (5′→3′)	Reverse primer (5′→3′)
vrrA[a]	CACAACTACCACCGATGGCACA	GCGCGTTTCGTTTGATTCATAC
vrrB1[a]	ATAGGTGGTTTTCCGCAAGTTATTC	GATGAGTTTGATAAAGAATAGCCTGTG
vrrB2[a]	CACAGGCTATTCTTTATCAAACTCATC	CCCAAGGTGAAGATTGTTGTTGA
vrrC1[a]	GAAGCAAGAAAGTGATGTAGTGGAC	CATTTCCTCAAGTGCTACAGGTTC
vrrC2[a]	CCAGAAGAAGTGGAACCTGTAGCAC	GTCTTTCCATTAATCGCGCTCTATC
CG3[a]	TGTCGTTTTACTTCTCTCTCCAATAC	AGTCATTGTTCTGTATAAAGGGCAT
pXO1[a]	CAATTTATTAACGATCAGATTAAGTTCA	TCTAGAATTAGTTGCTTCATAATGGC
pXO2[a]	TCATCCTCTTTTAAGTCTTGGGT	GTGTGATGAACTCCGACGACA
bams01[b]	GTTGAGCATGAGAGGTACCTTGTCCTTTTT	AGTTCAAGCGCCAGAAGGTTATGAGTTATC
bams03[b]	GCAGCAACAGAAAACTTCTCTCCAATAACA	TCCTCCCTGAGAACTGCTATCACCTTTAAC
bams05[b]	GCAGGAAGAACAAAAGAAACTAGAAGAGCA	ATTATTAGCAGGGGCCTCTCCTGCATTACC
bams07[b]	GAATATTCGTGCCACCTAACAAAACAGAAA	TGTCAGATCTAGTTGGCCCTACTTTTCCTC
bams13[b]	AATTGAGAAATTGCTGTACCAAACT	CTAGTGCATTTGACCCTAATCTTGT
bams15[b]	GTATTTCCCCCAGATACAGTAATCC	GTGTACATGTTGATTCATGCTGTTT
bams21[b]	TGTAGTGCCAGATTTGTCTTCTGTA	CAAATTTTGAGATGGGAGTTTTACT
bams22[b]	ATCAAAAAATTCTTGGCAGACTGA	ACCGTTAATTCACGTTTAGCAGA
bams23[b]	CGGTCTGTCTCTATTATTCAGTGGT	CCTGTTGCTCCTAGTGATTTCTTAC

Locus	Forward primer (5′→3′)	Reverse primer (5′→3′)
bams24[b]	CTTCTACTTCCGTACTTGAAATTGG	CGTCACGTACCATTTAATGTTGTTA
bams25[b]	CCGAATACGTAAGAAATAAATCCAC	TGAAAGATCTTGAAAAACAAGCATT
bams28[b]	CTCTGTTGTAACAAAATTTCCGTCT	TATTAAACCAGGCGTTACTTACAGC
bams30[b]	GCATAATCACCTACAACACCTGGTA	CAGAAAATATTGGACCTACCTTCC
bams31[b]	GCTGTATTTATCGAGCTTCAAAATCT	GGAGTACTGTTTGTTGAATGTTGTTT
bams34[c]	CAGCAAAATCAATCGAATCAAA	TGTGCTAAATCATCTTGCTTGG
bams44[c]	GCGAATTAATTGCTCCTCAAAT	GCACTTGAATATTTGGCGGTAT
bams51[c]	ATTTCCTGAAGCAGGTTGTGTT	TGCATCTAACAATGCAGAACAA
bams53[c]	GAGGTGTGTTAGGTGGGCTTAC	CATATTTTCACCTTAATTTTGGAAG

Notes: [a] Keim et al. 2000; [b] Le Flèche et al. 2001; [c] Lista et al. 2006

Results. ***Chromosomal and plasmid marker analysis and canonical SNP typing.*** *B. anthracis* chromosomal and pXO1 plasmid markers were detected in all 12 DNA samples. However, only 5 out of 12 tested strains were positive for the pXO2 plasmid marker (Table 4).

Table 4 — The list of 25 published *B. anthracis* VNTR markers

#	Sample	Chromosome *dhp61*	pXO1 *pagA*	pXO2 *capC*
1	BA-C-10-Cher	+	+	+
2	BA-D-12-Mel	+	+	+
3	BA-C-12-Sm	+	+	+
4	55 VNIIVViM	+	+	-
5	M-71	+	+	+
6	K-79-Z	+	+	-
7	SB	+	+	-
8	55	+	+	-
9	Tsenkovski-II	+	+	+
10	STI	+	+	-
11	Sterne 34F2	+	+	-
12	UA-07	+	+	-

Next, canSNP assays were performed to examine the phylogenetic position and genetic diversity of Ukrainian *B. anthracis* isolates. Allelic states denoted as 'ancestral' or 'derived' were scored in Table 5. No allelic discrepancies (contradictions) were observed.

Table 5 — Results of canSNP typing for samples from Ukrainian strains

<table>
<tr><th>#</th><th>Sample</th><th>A.Br.001</th><th>A.Br.002</th><th>A.Br.003</th><th>A.Br.004</th><th>A.Br.005</th><th>A.Br.006</th><th>A.Br.008</th><th>A.Br.007</th></tr>
<tr><td>1</td><td>BA-C-10-Cher</td><td rowspan="12">Ancestral</td><td rowspan="12">Ancestral</td><td rowspan="12">Ancestral</td><td rowspan="12">Ancestral</td><td rowspan="12">Derived</td><td rowspan="12">Ancestral</td><td rowspan="12">Derived</td><td rowspan="12">Ancestral</td></tr>
<tr><td>2</td><td>BA-D-12-Mel</td></tr>
<tr><td>3</td><td>BA-C-12-Sm</td></tr>
<tr><td>4</td><td>55 VNIIVViM</td></tr>
<tr><td>5</td><td>M-71</td></tr>
<tr><td>6</td><td>K-79-Z</td></tr>
<tr><td>7</td><td>SB</td></tr>
<tr><td>8</td><td>55</td></tr>
<tr><td>9</td><td>Tsenkovski II</td></tr>
<tr><td>10</td><td>STI</td></tr>
<tr><td>11</td><td>Sterne 34F2</td></tr>
<tr><td>12</td><td>UA-07</td></tr>
</table>

All strains grouped into the A.Br.008/009 SNP-clade, which belongs to the major 'A' branch of *B. anthracis*. This clade is also known as Trans-Eurasian subgroup, which is spread across Europe, the Middle East, and part of Asia, including China.

MLVA-typing. For more detailed phylogenetic characterization of Ukrainian samples, MLVA-25 analysis was conducted (Fig. 2). The majority of the Ukrainian *B. anthracis* strains, namely BA-C-10-Cher, BA-D-12-Mel, BA-C-12-Sm, 55 VNIIVViM, K-79-Z, SB, 55, STI, Sterne 34F2, and UA-07, were similar to strains from Bulgaria, described by Antwerpen et al. (2011). These strains form a unique sub-cluster within the A1.a lineage which is different from clusters of strains from geographically neighboring regions, such as Turkey, Georgia, Albany or Italy. Conversely, two samples had different genotypes which were similar to Italian (M-71) and Spanish strains (Tsenkovski-II). The preliminary results indicate that the pXO2-negative strains (55 VNIIVViM, K-79-Z, SB, 55, STI, Sterne 34F2 and UA-07) might be related to Russian vaccine strains. Based on these results, MLVA-tree was built (Fig. 2). Thus, the Ukrainian strain designated Sterne 34F2 is not a Sterne vaccine derivative (belonging to SNP group A.Br.001/002) but is most likely a relative to the STI-vaccine strain (A.Br.008/009).

Discussion. The analysis of a small set of canonical SNPs is a fast way to determine the major clonal sublineages of *B. anthracis* when assay costs are essential issues. The original setup described by Van Ert et al. (2007) required the use of 26 sequence-specific TaqMan minor groove binder (MGB) probes. This setup is quite expensive for laboratories where only a few strains have to be typed per year.

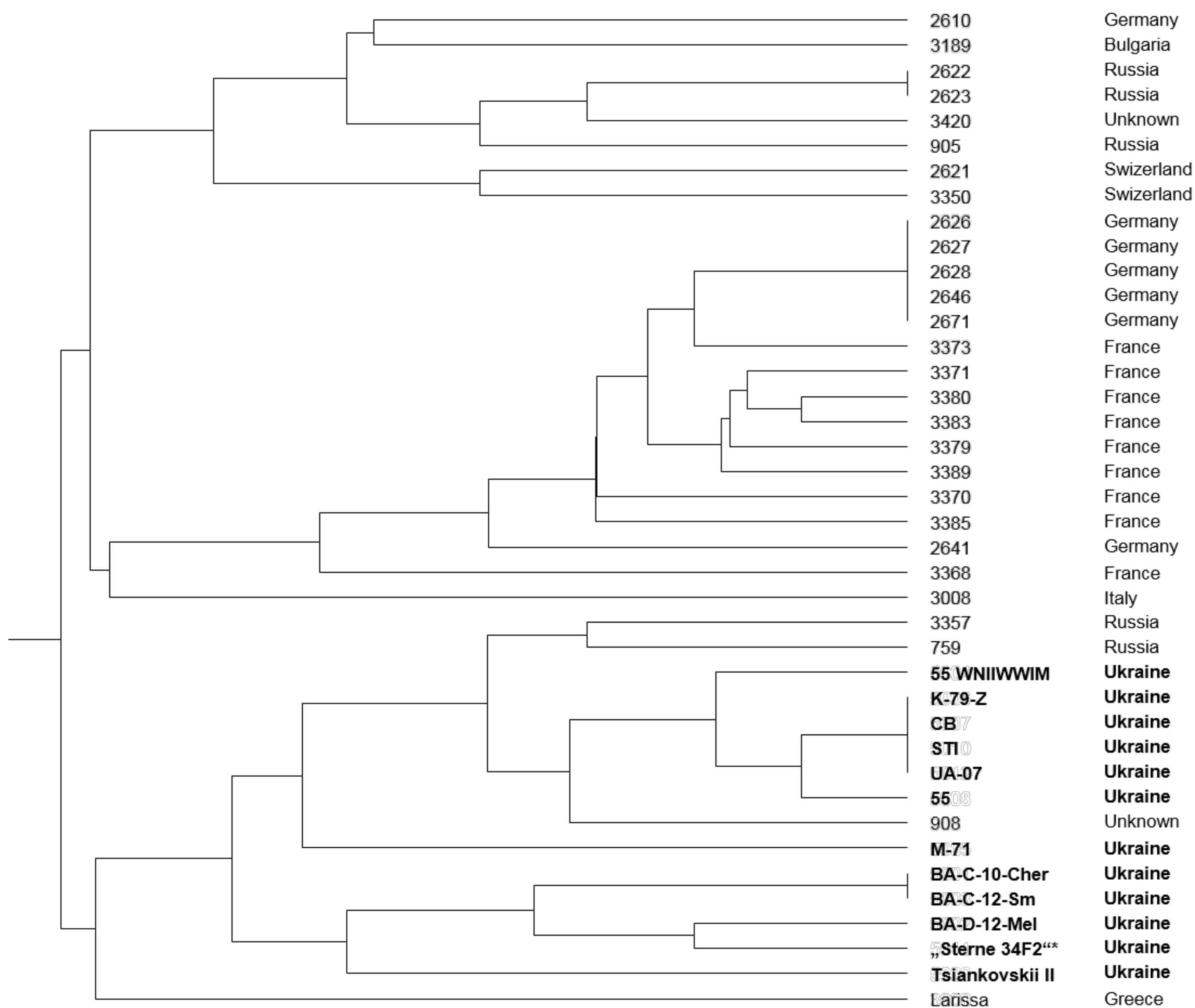

Figure 2. Dendrogram based on multi-locus variable-number tandem repeat analysis (MLVA-25) of Ukrainian and related isolates (numbers and letters) with countries of origin. A categorical coefficient was used for cluster analysis by the unweighted pair group method with arithmetic mean (UPGMA); * — incorrect name designation

The development of cheaper alternatives for interrogating canSNPs would increase access to these important phylogenetic markers for a larger number of laboratories. HRM is an attractive method, as it is simpler and cheaper than alternative approaches. HRM is a two-step, closed-tube assay that has high discriminatory power. The HRM assay requires only a few hours for each run, including follow-up data analysis, on a suitable PCR-instrument and can be performed in reaction volumes of less than 10 μl, reducing de facto the cost per analysis.

Over the recent years, significant research efforts have been undertaken to develop appropriate genotyping methods for differentiation of diverse *B. anthracis* strains. The currently available methods take advantage of tandem repeat polymorphisms or single base variations. A typing strategy relying on a combination of genetic markers that are progressively less stable but have increasing resolving power (SNP, VNTRs, including SNRs) has been recommended (Keim, 2004). In this system canSNPs typing is used to establish strain placement within broad phylogenetic groups followed by genotyping using MLVA. SNPs are evolutionary stable DNA signatures with low mutation rates (10^{-10} changes per nucleotide per generation) and only two allelic states. In addition, canSNPs typing can be applied to very low amounts of DNA, and/or degraded DNA, which can be essential in a forensic context (Birdsell et al., 2012). VNTR loci are genomic regions with higher mutational rate (ranging from $<10^{-5}$ to $>10^{-4}$ insertion-deletion mutations per generation) and typically higher numbers of possible allelic states (Keim, 2004). Notably, in contrast to canSNP-states, the number of VNTR repeats is not an indicator of

phylogenetic distance. Instead, these are just categorical differences useful for additional differentiation of strains harboring the same canSNP type.

The infrequent occurrence of anthrax in the country of Ukraine is likely caused by a heterogeneous population of *B. anthracis*. This population is phylogenetically composed of at least two different canSNP groups of the world-wide dominating A-branch of the pathogen. While one group might stem from environmental recovery of live vaccine strains used in Ukraine (or the former Soviet Union in the past) the other one, A.Br.008/009 is likely the autochthonous one that could be enzootic as indicated by the presence of related strains in countries of southeastern Europe in relatively close geographical vicinity to Ukraine.

Conclusions. The present work establishes the first preliminary picture of the genetic diversity of *B. anthracis* in Ukraine and provides valuable data sets for future epidemiological or forensic studies. This work might be a primer for a more detailed database describing the genetic landscape of *B. anthracis* diversity in Ukraine. It will then be possible to conduct future epidemiological and epizootological studies as well as for deriving bioforensic hypotheses on the origin of strains with unclear origin.

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