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SCREENING OF POSSIBLY ANTHRAX-CONTAMINATED BURIAL SITES IN EASTERN AND SOUTHERN UKRAINE

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Aim. The aim of this study was to screen soil samples of 17 anthrax burial sites in Eastern and Southern Ukraine for the presence of *B. anthracis.* **Methods.** Soil samples were collected from anthrax grave sites located in Kharkiv, Sumy and Mykolaiv regions (diseased animals dated from 1946 to 2003). Isolation of *B. anthracis* from collected soil samples was performed with the GABRI method. From single colonies without hemolysis, that were inactivated with peracetic acid- containing 2 % Terralin PAA solution, DNA was extracted and analyzed by qPCR for the presence of chromosomal marker *dhp61,* as well as the markers *pagA* and *capC* located on virulence plasmids pXO1 and pXO2, respectively. **Results.** Eleven field trips were conducted from July, 2016 to October, 2018 in which 369 soil samples from 17 burial sites in Kharkiv, Sumy and Mykolaiv oblasts were collected from different depths of presumed anthrax carcass sites. In most cases (12 out of 17 cases), the current status of these burial sites was deteriorated and not properly accounted for. It was possible to obrain viable *B. anthracis* isolate was obtained from 50 cm depth at the grave site near Koviagy village, Valky district, Kharkiv region (49.92373°N, 35.48951°E). This isolate was named KhR/VD/Kov2-2-05-3 and deposited in the Collection of Animal Infectious Pathogens of the National Scientific Center "Institute of Experimental and Clinical Veterinary Medicine", Kharkiv, Ukraine. The contamination level of soil at the isolation site reached about 10^4 CFU per g as determined by plate counting. qPCR analysis of this isolate identified both the *dhp61 B. anthracis* chromosomal and the *pagA* virulence plasmid marker. However, the plasmid pXO2 marker, required for capsule-formation could not be detected. **Conclusions.** The anthrax burial sites were created between the 1920s and 1960s, however, only approximate locations could be found and demarcated. In most cases the status of the sites was unsuitable for sampling. Nevertheless, isolation of *B. anthracis* in one case in the Valky district shows that old anthrax burial sites (13.500 exist in Ukraine) still pose a risk as potential source of the infection and therefore require more attention and surveillance, for which a surveillance plan will be developed.

Key words: anthrax, *B. anthracis,* animal burial sites, soil, spores, polymerase chain reaction, DNA.

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INTRODUCTION

Anthrax is a zoonotic disease to which mainly grazing herbivores, but also omnivores, carnivores and human are susceptible. Under adverse conditions the causative pathogen *Bacillus anthracis* is able to form spores that may remain viable in the environment, especially in soil, for many decades. Due to this biological feature and its high virulence, this pathogen is still of great scientific and epidemiological importance in many areas of the world. Each year, more than a million (mainly wild) animals and about 20,000 people in 82 countries around the world get infected with anthrax (Logvin et al, 2017). This pathogen is easy to multiply and has a high recalcitrance in the environment. Laboratory produced anthrax spores of pathogenic strains therefore represent a potential threat related to bioterrorism, as they are easy to produce and store (Hoffmaster et al, 2001; Keim et al, 2004; Eitzen et al, 1997).

Over the years many (24 954) enzootic and epizootic outbreaks of anthrax have been recorded in Ukraine, although in the last 15 years more sporadically (Koro-

tych et al, 1976; Skrypnyk et al, 2012; Bezimennyi et al, 2014; Bobylyova and Mukharska, 2001; Bobylyova and Mukharska, 2002; State Scientific and Research Institute of Laboratory Diagnostics and Veterinary and Sanitary Expertise [SSRILDVSE], 2014). In recent years, the epizootic situation concerning anthrax in Ukraine has decreased (largely due to the reduction in total numbers of livestock). However, there is still a significant risk of new outbreaks, mainly due to the large number of old animal burial sites (13,500) and their unresolved current status. It was repeatedly established by researchers from Russia (Dugharzhapova et al, 2016; Shishkova et al, 2011), Albania (Peculi et al, 2015), Brazil (Salgado, 2020) and Italy (Fasanella et al, 2015, Braun et al, 2015), that these burial sites of anthrax infected animal carcasses pose a particular risk as natural reservoir of this pathogen. In addition, there are most likely unknown soil foci of the pathogen originating from herding or war (World War II) casualties. A hypothesis therefore can be that these burial sites are important extant sources of the pathogen. Reported facts of increased informal-illegal slaughtering of infected animals and eating of illegally slaughtered meat, i.e. anthrax outbreak in Georgia (Kracalik et al, 2014), as well as recent case Minyailivka village, Odesa region, Ukraine (Ministry of Health of Ukraine, 2018; 112 Ukraine, 2018), serve as proof of particular danger posed by uncontrolled anthrax foci. Therefore, these sites require careful attention and constant monitoring by state laboratory facilities according to governmental regulatory guidelines. In particular, data on epizootic anthrax foci must be documented in special district register of the epizootic condition, which should be permanently stored in the district veterinary department, as well as marked on the epizootic map of the district, indicating the date, number of sick and dead animals, the exact location of the infection. Also, fencing and compliance with appropriate sanitary condition of anthrax burial sites and biothermal pits should be properly controlled. Strict observance of veterinary and sanitary requirements during carrying out agricultural, construction and other works, associated with the movement of soil are also the key points to control the disease. (Ministry of Agriculture of Ukraine & State Department of Veterinary Medicine, 2000).

In the view of the above, the goal of this work was to carry out a limited survey of 17 anthrax burial sites in Eastern and Southern Ukraine.

MATERIALS AND METHODS

For this research, 369 soil samples were taken from 17 anthrax animal burial sites in Kharkiv, Sumy and Mykolaiv oblasts (regions) dug in 1946–2003. The majority of burials (12 out of 17) were studied in Kharkiv region since, according to Skrypnyk et al. (2012), one of the biggest numbers of grave sites in the country are located in this region. Samplings in Mykolaiv region were conducted in close cooperation with specialists of Regional Laboratory Center in frames of the Cooperation Agreement between NSC "IECVM" and Mykolaiv RLC from 04.10.2017. We also collected samples in southern part of Sumy region since one of the biggest natural foci of anthrax was located in this area (Bezimennyi et al, 2014). Samples were collected together with specialists from the local district veterinary services with the permission of the State Veterinary and Phytosanitary Service of Ukraine (now – State Food and Consumer Service of Ukraine), the State National and Scientific Institute of Laboratory Diagnostics and Veterinary and Sanitary Expertise and the State Scientific and Control Institute of Biotechnology and Microorganism Strains (#15-9-1-18/27215 from 11.20.2015). Data on the locations of anthrax burial sites in the north-east of Ukraine was kindly provided by the Main Departments of Veterinary Medicine in Kharkiv, Sumy and Mykolaiv oblasts.

Sampling. All samplings were performed using personal protective equipment (PPE) (overalls, respiratory masks, goggles, nitrile gloves and protective shoes). At each burial site, several samples were taken, the average distance between dig or drill holes being 2–3 m. From each point, one soil sample was withdrawn from upper ground layer (0–10 cm); samples from deeper soil layers (one sample from each 10 cm of soil layer, 5–10 soil samples from each spot) were collected using a 1 m-length metal so-called Dr Pürckhauer's Soil Sample Drill for Medium to Heavy Soils. If necessary, a metal extension of 1 m was attached to the auger. The auger was forced into the ground using a hammer of 3 kg weight with plastic stoppers. Once the required depth was reached, a column of earth was punched out of the ground and the soil was filled into separate 50 ml plastic tubes from a depth of 10 cm each. In total of 6–11 samples from each point were placed in separate zipper plastic bags, which, in turn, were folded into plastic containers for transportation. After sampling from each collection point, the auger was treated with a fresh disinfectant solution (2 % terralin PAA solution, Schülke, France), and was washed and lubricated after each field work. Upon completion of field

works, all personal protective equipment and disposable materials used were incinerated.

Semi-selective isolation of B. anthracis *from soil samples.* To detect *B. anthracis* spores in collected samples, the GABRI method (Fasanella et al, 2013) was used. In contrast to the classical one, this method significantly reduces bacterial and fungal contaminants from cultivated samples, which inhibit the growth of *B. anthracis* (Fasanella et al, 2013). Semi-selective blood agar medium (Carl Roth, Germany) with the addition of 5 % sterile defibrinated sheep blood and antibiotics (polymyxin B – 20 mg/ml, trimethoprim – 25 μg/ml and sulfamethoxazole – 40 μg/ml) was used for microbiological studies. All studies were performed in a Thermo Electron MSC-Advantage™ Class II Biological Safety Cabinet (Thermo Scientific, USA) according to the GABRI-method protocol (Fasanella et al, 2013). Supernatant from each soil sample was inoculated on three Petri dishes with blood agar. After 36 hours of cultivation at 37 °C, non-hemolytic colonies were re-inoculated onto Columbia agar (Merck-Millipore, USA) medium prepared with the addition of 5 % sheep blood and antibiotics (see above). For each sample, colonies from three blood agar plates were re-inoculated on one separate plate with Columbia agar divided into three sectoral parts and incubated, aerobically, for 24 hours at 37 °C. The viability of cultures and the contamination of soil with *B. anthracis* spores were determined using quantitative indices such as the number of CFU per 1 g of soil and the average number of colonies per one Petri dish. In order to prepare positive control of GABRI, 7.5 grams of soil collected from garden in the city of Kharkiv were tested for potential contamination with *B. anthracis* spores. After confirmation of spores' absence, this sample was artificially contaminated with the suspension containing 500 spores of *B. anthracis* Sterne 34F2 vaccine strain.

Sample preparation for qPCR. Non-hemolytic colonies on Columbia agar with a diameter of more than 1 mm after 24 hours of cultivation at 37 °C, were further analyzed by real-time PCR (qPCR). For this purpose, the growth of single colonies was transferred with a disposable loop into individual plastic tubes, subsequently inactivated with 2 % terralin PAA solution for 30 minutes at room temperature, centrifuged for 2 minutes at 6000 g, washed twice with PBS, centrifuged again and resuspended in 50 μl of nuclease-free Milli-Q water. DNA Obtained samples were diluted 1:10 and aliquoted into several microtubes (10 μl to each tube). DNA extracted from the culture of the vaccine strain Sterne 35F2 was used as positive control. A negative control containing 5 μl water instead of bacterial DNA was included in each PCR run. After qPCR studies, aliquots were stored at minus 70 °C.

qPCR. Specific primers (TIB MolBiol, Germany) were used for detection of anthrax-specific chromosomal and plasmid markers as described by Antwerpen et al (2008), Liang et al (2016) and Hadjinicolaou et al (2009). Primers as well as reference DNA of pathogenic Ames 3013 strain and the Sterne 34F2 attenuated non capsule forming (pXO2 plasmid negative) strain of *B. anthracis* were kindly provided by the Bundeswehr Institute of Microbiology as part of the international "Ukrainian-German programme for biosecurity and zoonotic risk management at the EU external borders". The samples were analyzed for the presence of the specific chromosomal marker *dhp61* (forward 5'CGT AAG GAC AAT AAA AGC CGT

Table 1. Compositions of reaction mix components used for all four primer pairs

Reagent	Amount per 1 reaction, μl			
	dhp61 chromosomal marker	*pagA* (pXO1 plasmid marker)	*capC* (pXO2 plasmid marker)	*gyrA* (*Bacillus* multispecies marker)
Water, nuclease free	14.38	12.30	13.25	14.38
10× PCR buffer	2.5	2.5	2.5	2.5
25мМ Mg^{2+}	1.5	3.0	2.0	1.5
dNTP mix	0.5	0.5	0.5	0.5
forward primer	0.5	0.75	0.25	0.5
reverse primer	0.5	0.75	1.25	0.5
Taq-polymerase, 5 U/μl	0.13	0.2	0.25	0.13
Template DNA	5	5	5	5

TGT3'; reverse 5'CGA TAC AGA CAT TTA TTG GGA ACT ACA C3'; probe 6FAMTGC AAT CGA TGA GCT AAT GAA CAA TGA CCC TTAMRA) (Antwerpen et al., 2008). Later *dhp61*-positive samples were tested for the presence of the pXO1 plasmid marker *pagA,* (forward 5'GTA CAA GTG CTG GAC CTA CG3'; reverse 5'CAC TGT ACG ATC AGA AGC C3'; probe 6FAMACC GTG ACA ATG ATG GAA TCC CTG ABBQ) (Xudong Liang et al., 2016) and the pXO2 plasmid marker *capC,* (forward 5'CCT GCA GGT TTA GTT GTA CCT3'; reverse 5'ACC TGT AAT TAG CGT TGC CG3'; probe 6FAMAGC ACT CGT TTT TAA TCA GCC CGTBBQ) (Hadjinicolaou et al., 2009). *B. anthracis* isolates were differentiated from other bacilli by detecting a *Bacillus* multispecies *gyrA* marker using primers described by Hurtle et al. (2004) (forward 5'ATG TCA GAC AAT CAA CAA CAA GC3'; reverse 5'GCA ATG AGT GTT ATC GTT CTC G3'; probe 6FAMTAT TAG CCA TGA ATC GCG TBBQ). The reaction mix was prepared using *AmpliTaq Gold* reagents manufactured by Applied Biosystems (USA). For each testing, separate reaction mixtures were prepared (table 1) and, as for the detection of *pagA* and *capC,* concentrations of components were used according to our previous qPCR validation results (Beloyvan et al, 2019; Biloivan et al, 2019a).

DNA amplification was performed on a Fast 7500 Real-time PCR system manufactured by Applied Biosystems (table 2). Amplification of *dhp61* and *gyrA* fragments were performed as described by Antwerpen et al (2008) and Hurtle et al. (2004), respectively. For *pagA* and *capC* amplification, annealing temperatures were previously validated (Beloyvan et al, 2019; Biloivan et al, 2019a).

For further DNA extraction, *B. anthracis dhp61*-positive isolates were re-inoculated into liquid LB-medium and harvested. For this purpose, liquid cultures were aerobically cultivated for 24 hours at 37 °C and centrifuged for 2 minutes at 6000 g. Then, broth supernatant was removed from the tube, bacterial pellet was inactivated with 2 % terralin PAA solution, washed with PBS twice and resuspended in Milli-Q water as described above. Subsequently, DNA was extracted using InnuPREP DNA Mini Kit manufactured by AnalytikJena (Germany). Obtained DNA samples were re-examined for the presence of chromosomal and plasmid markers of anthrax pathogen. Broth cultures of PCR-positive samples were transferred on Microbank™ cryovial systems and stored at minus 70 °C.

RESULTS

During the period from July 2016 to October 2018, 11 field expeditions to anthrax cattle burial sites were conducted in Kharkiv, Sumy and Mykolaiv oblasts. Burials are located near the villages:

– Zavody, Izum rayon (district), Kharkiv oblast, (49.176°*N*, 36.991°*E;* 1960);
– Kostiv (49.51444°*N*, 35.39013°*E;* 1953), Dobropillya (49.96807°*N*, 35.71525°*E;* 1961), Koviagy (49.92373°*N*, 35.48951°*E*; 1964), Snizhkiv (49.47675°*N*, 35.32195°*E;* 1959), Sharivka (49.917277°*N*, 35.786629°*E;* 1958), Valky rayon, Kharkiv oblast;
– Verkhnya Samara (48.38375°*N*, 36.39876°*E;* 1946), Vilne-1 (48.80983°*N*, 36.62448°*E;* 1951), Blyzniuky rayon, Kharkiv oblast;
– Nyzhche Solone (49.18574°*N*, 37.40158°*E;* 1956), Gorokhuvatka (49.18828°*N*, 37.30499°*E;* 1961), Borivska Andriivka (49.35194°*N*, 37.67881°*E*; 1971), Klymivka (49.22170°*N*, 37.41113°*E;* 1986), Borova rayon, Kharkiv oblast;
– Timiryazivka (50.96°*N*, 34.34°*E;* 1994), Bilopillya rayon, Sumy oblast;
– Sinyushin Brid (48.09466°*N*, 30.48597°*E;* 1946), Stanislavchik (48.07595°*N*, 30.47147°*E;* no data), Pervomaysk rayon, Mykolaiv oblast;

Table 2. Amplification parameters for all four DNA fragments

Stage	Amplification parameters				Cycles
	dhp61	*pagA*	*capC*	*gyrA*	
Initialization	95 °C – 5 min	95 °C – 5 min	95 °C – 5 min	95 °C – 5 min	1
Denaturation	95 °C – 15 sec	95 °C – 15 sec	95 °C – 15 sec	95 °C – 15 sec	40
Annealing	**55 °C – 20 sec**	**62 °C – 20 sec**	**60 °C – 20 sec**	**55 °C – 20 sec**	
Elongation	72 °C – 40 sec	72 °C – 40 sec	72 °C – 40 sec	72 °C – 40 sec	
Final elongation	72 °C – 1 min	72 °C – 1 min	72 °C – 1 min	72 °C – 1 min	1

– Malakhovo (46.54507°*N,* 31.21481°*E;* 1959), Dmytrivka (46.56664°*N*, 31.20349°*E;* 2003), Berezanka rayon, Mykolaiv oblast.

In total, 369 soil samples were collected of 17 locations only (1 in Sumy, 4 in Mykolaiv and 12 in Kharkiv regions).

The conditions of the burial sites were unsatisfactory in most of cases. 12 out of 17 locations did not have distinct warning signs and were not fenced. In addition, the locations of eight of these sites were inaccurate, and 11 of them were located nearby crop fields (50 meters and closer), posing a significant risk of further spread of contamination during soil cultivation. According to Hugh Jones & Blackburn (2009), *B. anthracis* spores longer stay viable in humus soils rich with calcium, phosphorus and magnesium, than in sands or loams. The soils in Kharkiv and Sumy regions are predominantly humus-rich. Loamy soils were only identified in burial sites near the villages of Kostiv and Dobropillya in Valky rayon and in the nearby village Nyzhche Solone, where the surface ground layer was humus-rich and the deeper layers loamy. In contrast to the Eastern regions of Ukraine, loamy soils predominated in sampling areas in the Mykolaiv oblast. Chernozem soil was only found at the burial site nearby Stanislavchyk village in the Pervomaysk district.

Nevertheless, *B. anthracis* could be isolated from a soil sample taken from 50 cm depth at the place of mass burial of anthrax infected animals nearby the village of Koviagy, Valky rayon, Kharkiv oblast (49.92373°*N*, 35.48951°*E*). According to the documents, eight cattle and one pig carcass, which died from anthrax were buried at this site in 1964. However, according to locals, anthrax carcasses were still buried at this site at least four times in the period from 1953 to 1964. This grave site is located in a depression, 20 meters long, 10 meters wide and 5 meters deep, located on the edge of a crop field near the forest plantation. Numerous bone fragments of animals (cattle, pigs) have also been found in this deepening. No demarcations, i.e. fencing or warning signs of this anthrax burial site were observed.

According to GABRI results, three out of 19 samples collected at the grave site near Koviagy village were positive. In positive samples taken at depths of 10, 50 and 80 cm (marked as KhR/VD/Kov2-2-01-1, KhR/VD/Kov2-2-05-3 and KhR/VD/Kov2-2-08-2, respectively), greyish rough and irregular-shaped colonies without hemolysis were observed (Fig. 1). Also,

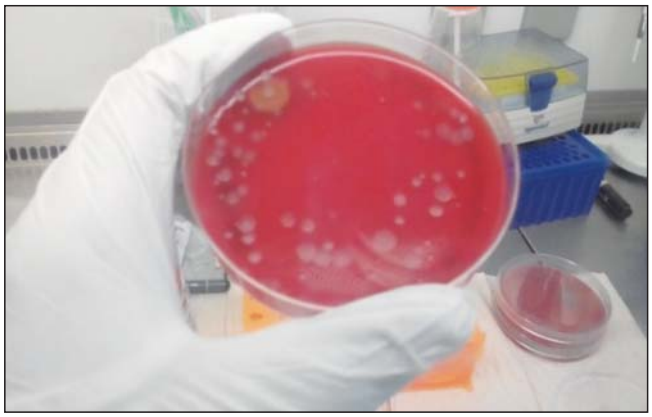

Fig. 1. 36-hour old *B. anthracis* culture isolated from Koviagy soil sample on blood agar: positive colonies are rough, greyish and non-hemolytic

the anthrax-typical beaten-egg-white effect appeared when growth of the colony was lifted with an inoculation loop. These colonies were inoculated on Columbia agar with the addition of 5 % defibrinated sheep blood and incubated for 24 hours at 37 °C.

Quantitatively, the contamination level of the soil samples was about 104 CFU/g or an average of 15, 68 and 62 colonies per Petri dish for KhR/VD/Kov2-2-01-1, KhR/VD/Kov2-2-05-3 and KhR/VD/Kov2-2-08-2, respectively. Colonies with similar morphology to *B. anthracis* were also obtained from the following soil samples:

– Borivska Andriivka, Borova rayon, Kharkiv oblast – 5 samples;

– Dobropillya, Valky rayon, Kharkiv oblast –5 samples;

– Gorokhuvatka, Borova rayon, Kharkiv oblast – 1 sample;

– Malakhove, Berezanka rayon, Mykolayiv oblast – 4 samples;

– Sharivka, Valkiv district, Kharkiv region – 2 samples.

Isolated colonies from these samples were also spread out on Columbia agar plates with the addition of 5 % defibrinated sheep blood and incubated for 24 hours at 37 °C. A total of 14 colonies were isolated and inactivated from samples from Borivska Andriivka, 8 – from Dobropillya, 2 – from Gorokhuvatka, 9 – from Malakhovo and 5 – from Sharivka and analysed by qPCR.

The chromosomal *B. anthracis* marker *dhp61* was detected only in KhR/VD/Kov2-2-05-3 isolate from Koviagy. The ct value was 22.13 (Fig.2).

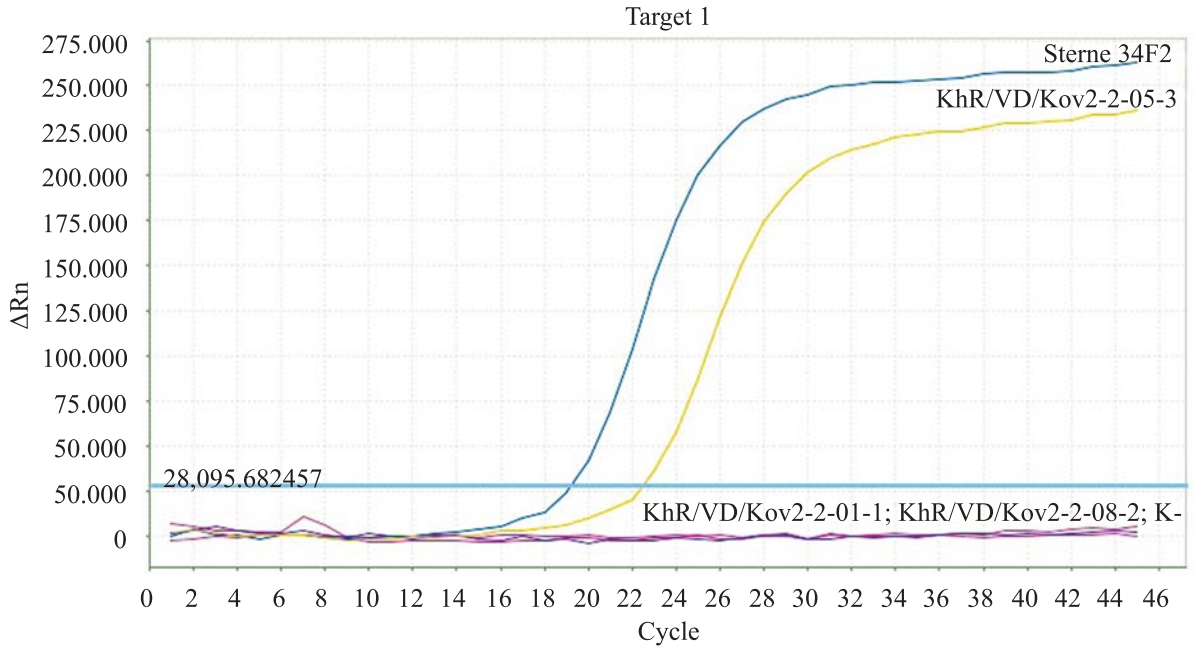

Fig. 2. Positive qPCR result of *B. anthracis dhp61* chromosomal marker in DNA samples from Koviagy. DNA extracted from the culture of the vaccine strain Sterne 34F2 was used as positive control, the negative control is marked as K-

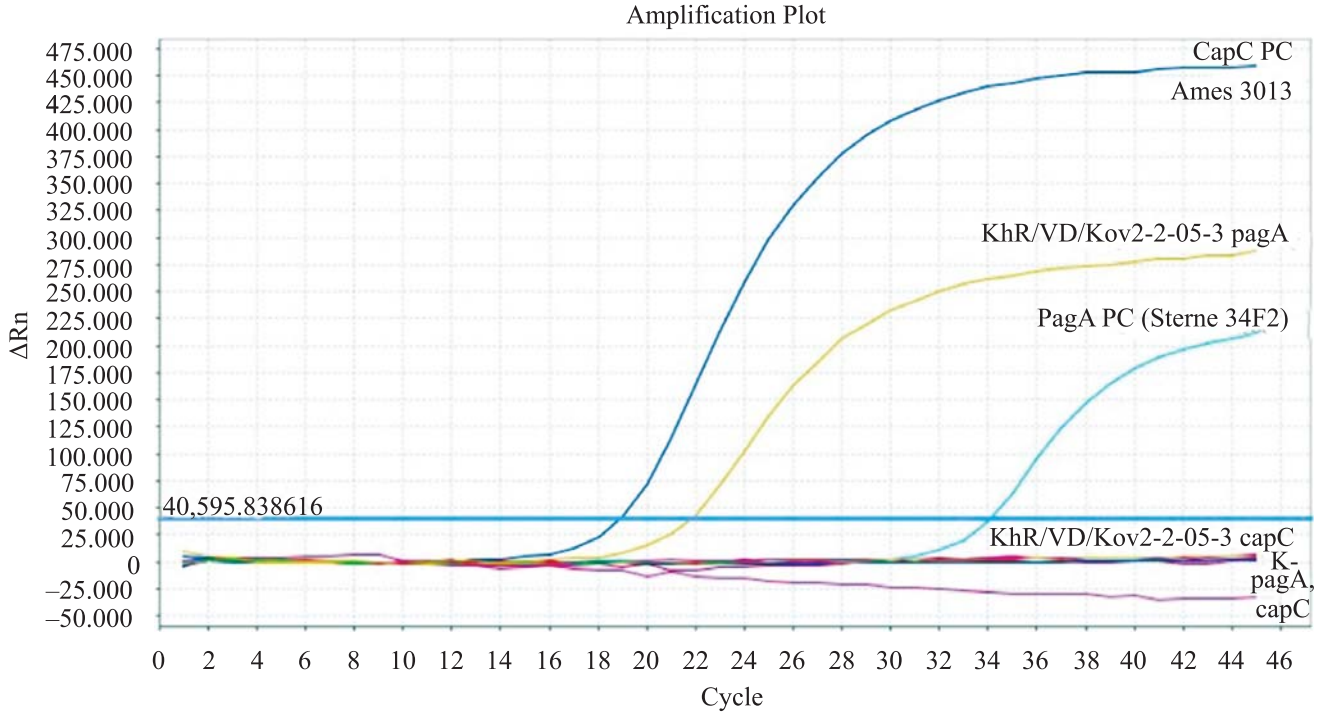

Fig. 3. Detection of the *pagA* virulence plasmid marker in qPCR. DNA of *B. anthracis* strain Sterne 34F2 was used as positive control

Furthermore, the *dhp61* positive isolate KhR/VD/Kov2-2-05-3 was tested for the presence of *pagA* and *capC* markers, which are specific for pXO1 and pXO2 anthrax plasmids, respectively (Fig. 3).

As shown in Figure 3, the KhR/VD/Kov2-2-05-3 isolate contains only the pXO1 plasmid marker *pagA*. Therefore, it was characterized as a possible non-virulent variant of *B. anthracis*.

Analyses of other 38 isolated strains failed to detect the chromosomal marker *dhp61*. In order to determine their relationship to the genus *Bacillus*, these samples were analysed for the multispecies *gyrA* housekeeping

gene (according to Hurtle et al, 2004). Samples with a DNA concentration greater than 100 ng/µl were diluted (Table 3).

Thus, 34 positive samples were identified as *B. cereus sensu lato* bacilli, i.e. bacteria that are genetically closely related to *B. anthracis.*

DISCUSSION

According to archival data from regional laboratory centers, 13,500 animal grave sites with a history of anthrax are officially registered in Ukraine. A total of 144 anthrax animal burial grounds are located in Kharkiv oblast (State Institution "Kharkiv Regional Laboratory Center of the Ministry of Health of Ukraine", 2018). In addition, there are most likely many more, unknown soil foci that have been contaminated with the pathogen during the burial of animals at the beginning of the past century or after cattle movements during World War II. Due to the fact that these burial sites are the main reservoirs of the pathogen, they require special attention and constant monitoring of their condition by the state laboratory facilities. The unsatisfactory condition of most burials can be due to insufficient demarcation as well as poor or incorrect administration in the municipalities. Another important fact is that many cattle grave sites were dug more than 50 years ago. When these sites were established (1920s–1960s), there were no GPS satellite navigation systems. Information on their location was recorded in anthrax logs, which provided only approximate descriptions of the sites' location based on nearby landmarks. Over the years, the landscape has changed steadily, the terrain and vegetation have undergone major changes and most anthropogenic infrastructure has either seen major alterations or disappeared completely. In addition, many people have already died who were able to specify the exact location of these animal burial sites.

In addition, the soil type plays a significant role in the survival of *B. anthracis* spores. Soils with a high concentration of calcium, phosphorus and magnesium are more favorable for a longer survival of *B. anthracis* spores, whereas in sandy and loamy soils the survival of spores is significantly reduced (Hugh Jones & Blackburn, 2009). This may have been an influence of absence of *B. anthracis* in samples from animal burial sites with loamy soils (nearby Kostiv, Dobropillya, Nyzhche Solone, Sinyushin Brid, Malakhivka and Dmytrivka). However, Van Ness (1971) noted that anthrax outbreaks mainly occur in the dry summer months after the rainy season, typically in May-June. These climatic aspects and the fact that spores have a high flotation capacity, indicate that water plays an important role in the ecology of this pathogen. Rainwater that washes away the soil, tends to collect in the landscape depressions, leading to the accumulation of spores. This increases the likelihood of the animals becoming infected with

Table 3. The qPCR results of the multispecies *gyrA* marker from isolated colonies of soil samples

Sample	Dilution	ct value
Borivska Andriivka-1/1-1a	–	35.71
Borivska Andriivka-1/1-1b	–	37.13
Borivska Andriivka-1/1-1c	–	35.41
Borivska Andriivka-1/1-2	–	37.69
Borivska Andriivka-1/1-4	–	negative
Borivska Andriivka-1/2-2	–	32.76
Borivska Andriivka-1/3-1	–	37.14
Borivska Andriivka-1/3-2	–	35.79
Borivska Andriivka-1/3-3	–	38.81
Borivska Andriivka-1/3-4	–	35.90
Borivska Andriivka-1/4-1	–	35.76
Borivska Andriivka-1/4-2	1:20	negative
Borivska Andriivka-1/4-4	–	38.89
Borivska Andriivka-1/5-4	–	16.97
Dobropillya-1-01-1	–	35.58
Dobropillya-1-01-3	1:10	32.99
Dobropillya-1-05-2	1:15	36.52
Dobropillya-1-08-1	1:15	38.47
Dobropillya-1-08-3	1:5	36.48
Dobropillya-1-09-2	1:10	36.72
Dobropillya-1-09-3	–	negative
Dobropillya-1-10-3	–	negative
Gorokhuvatka-3-06-3a	–	21.77
Gorokhuvatka-3-06-3b	–	20.21
Malakhove-2-06-1/2	1:10	36.00
Malakhove-2-06-2/1	1:20	36.13
Malakhove-2-06-2/2	1:25	35.43
Malakhove-2-06-3/2	1:10	31.07
Malakhove-2-09-3/3	1:50	37.63
Malakhove-2-10-1/1	1:50	33.28
Malakhove-2-10-1/2	1:50	35.30
Malakhove-3-06-1/1	1:25	27.55
Malakhove-3-06-1/2	1:25	31.17
Sharivka-1-09-1/1	1:50	24.80
Sharivka-1-09-1/2	1:5	30.10
Sharivka-1-09-1/3	1:100	19.29
Sharivka-1-09-2/3	1:50	35.47
Sharivka-1-10-1/1	1:25	34.41

anthrax spores during grazing. However, it takes time and certain natural phenomena to create such secondary anthrax foci that can cause new infections in animal pastures (Van Ness, 1971, Cherkasski, 2002, Revich & Podolnaya, 2011). A typical example of such a depression is the burial site located nearby Koviagy village, Valky rayon, Kharkiv oblast. This burial is a large depression 20 meters long, 10 meters wide and 5 meters deep, located on the edge of an agricultural crop field in a forest plantation. The soil in this area is predominantly humus-rich. According to local veterinarians, burials of anthrax-infected animal carcasses have occurred here several times since 1964. Furthermore, it was possible to isolate *B. anthracis* from the soil sample taken at a relatively shallow depth (50 cm). Besides, numerous animal bone fragments could be observed in the area. It can be explained that animal carcasses could not be buried deep into the ground or ground layer could be partially washed out with rainwater, leaving carcass residuals on the surface.

Microbiologically, the causative agent of anthrax can be easily isolated from the environment and it grows well on solid media, in particular, on blood agar. However, the isolations of the pathogen from the soil are often accompanied by a considerable amount of other microbial flora. Contaminants such as fungi, bacteria and especially spore-forming saprophytic bacteria closely related to *B. anthracis (B. thuringiensis, B. cereus, B. mycoides* etc.) complicate the isolation of desired strains (Turnbull, 1999). In order to minimize the vegetative microflora, samples were heat-treated in a first step (Dragon & Rennie, 2001). However, the temperature increase is ineffective against spore-forming bacteria, making the use of selective nutrient media mandatory. Dragon and Rennie (2001) showed that the use of selective nutrient media plays a key role in the isolation of *B. anthracis* spores from environmental samples. PLET agar (Polymyxin – Lysozyme – EDTA – Thallous acetate) and CHRA (Anthracis Chromogenic Agar) are semi-selective media that can inhibit the growth of many saprophytic bacteria groups and stimulate bacilli growth (Marston et al., 2008). However, thallium acetate is highly toxic itself and its use requires strict work safety and environmental protective measures (Tomaso et al, 2006; Turnbull, 2008). As for CHRA, Marston et al, (2008) noticed in their studies, that it is less sensitive to *B. anthracis*, than PLET agar.

Although such classical methods for isolation of *B. anthracis* spores is quite complex and time-consuming compared to other molecular methods, including PCR, it is still considered to be one of the most sensitive methods for pathogen detection in environmental samples (Gulledge et al, 2010). Molecular techniques based on the extraction of *B. anthracis* DNA directly from environmental samples are often not sufficiently effective since *B. anthracis* spores are quite resistant to chemicals. Additionally, organic inhibitors present in the samples can significantly reduce the efficiency of DNA amplification (Ryu et al, 2003).

The GABRI method was used for microbiological investigations of soil samples. Its effectiveness in using more selective media as compared to the classical ones was experimentally demonstrated by Fasanella et al. (2013). This method allows a more efficient isolation of *B. anthracis* spores due to inactivation of vegetative (background) microflora, which can inhibit the growth of anthrax colonies on blood agar by heating samples in a water bath and by adding antibiotics to the culture medium (trimethoprim, sulfamethoxazole and polymyxin B), as well as Fosfomycin before cultivation. In addition, the washing steps with 5% Tween-20 allow the separation of bacterial spores from soil particles by breaking hydrophobic bonds. By applying this GABRI method, several environmental *B. anthracis* isolates could be successfully obtained by Fasanella et al. (2013). In our work, the appearance of rough greyish non-hemolytic colonies was observed after 36 hours of incubation on blood agar showing typical morphological characteristics of *B. anthracis*. qPCR results have shown that the obtained isolate is a possible avirulent *B. anthracis* variant, but we still need to do further studies on the one isolate to be sure about. Isolation of pathogenic STI isolates in Caucasus, Yakutia and Western Siberia were reported by Pisarenko et al (2019). Moreover, both pXO1 and pXO2 plasmids may be lost long storage within ground waters and also under certain other environmental or laboratory conditions (Turnbull et al, 1992; Marston et al, 2005). Thereby, in order to make more clear statements about origin of obtained isolate as well as to differentiate it precisely from other bacilli, it is necessary to use complex approaches, including state-of-art phylogenetic methods.

CONCLUSIONS

The successful isolation of live *B. anthracis* thus demonstrates the potentially long-lasting epizootic and epidemiological risk posed by biological active old anthrax animal burial sites. Further work in the surveillance of anthrax burial sites in Ukraine with microbiological isolation of *B. anthracis* cultures is needed. It can be the basis for more detailed further re-

search, which will give a complete data for the sources of anthrax in Ukraine, as well as allow to predict its spread. This will help to carry out more effective control measures when epizootics occur. Furthermore, to determine find out the origin of possible, previously unknown strains occurring in these sites, by comparing new isolates with the existing collection of Ukrainian and worldwide *B. anthracis* strains.

Adherence to ethical principles. All experiments described in this paper were non animal based.

Conflict of interest. Authors declare no conflict of interest.

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Скринінг сибіркових поховань тварин на Сході та Півдні України

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Мета. Провести скринінг зразків ґрунту з 17 сибіркових поховань Сходу та Півдня України на наявність в них спор *B. anthracis*. **Методи.** Зразки ґрунту для подальших досліджень відбирали з сибіркових худобомогильників, розташованих на території Харківської, Сумської та Миколаївської областей (поховання 1946–2003 рр.). Виділення спор *B. anthracis* з відібраних проб ґрунту проводили за методом GABRI. Поодинокі колонії без ознак гемолізу інактивували 2%-ним розчином терраліну з вмістом пероцтової кислоти та досліджували за допомогою ПЛР-РЧ на наявність специфічного хромосомного маркера *dhp61*, маркера *pagA* плазміди pXO1 і маркера *capC* плазміди pXO2. **Результати.** За період з липня 2016 р. по жовтень 2018 р. проведено 11 польових експедицій, під час яких відібрано 369 зразків ґрунту з 17 захоронень, потенційно контамінованих спорами сибірки, розташованих на території Харківської, Сумської та Миколаївської областей. В більшості випадків стан поховань є незадовільним, а їх облік не проводиться належним чином. З проби ґрунту, яку було відібрано на глибині 50 см на місці сибіркового худобомогильника поблизу селища Ков'яги Валківського району Харківської області (49.92373° пн.ш., 35.48951°сх.д.), виділено ізолят *B. anthracis* KhR/VD/Kov2-2-05-3, який зберігається в репозитарії вірусних та бактеріальних патогенів Національного наукового центру «Інститут експериментальної і клінічної ветеринарної медицини (м. Харків). Рівень контамінації зразків ґрунту, з яких було виділено ізолят, склав близько 104 КУО/г. За результатами ПЛР-РЧ ізолят є позитивним на наявність хромосомного маркера *dhp61* збудника сибірки, а також містить маркер *pagA* плазміди pXO1. Проте, у нього відсутня капсулоутворююча плазмідіа pXO2. **Висновки.** Більшість сибіркових поховань була створена в період між 1920 та 1960 рр. минулого століття. Однак, в районних журналах з реєстрації сибіркових поховань описано лише їхні приблизні місцезнаходження. В більшості випадків відбір зразків ґрунту був ускладненим через неможливість точного визначення розташувань цих поховань. Тим не менш, факт виділення ізоляту *B. anthracis* з ґрунту у Валківському районі Харківської області доводить серйозну загрозу старих сибіркових поховань трупів тварин як птенційних джерел захворювання, а тому вони потребують постійних скринінгових досліджень та нагляду.

Ключові слова: сибірка, худобомогильники, ґрунт, спори, полімеразна ланцюгова реакція, ДНК.

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