Part 3. Biosafety and biosecurity

UDC: 619:616.98:579.852.11:614.443(560+477)

MODERN TURKISH APPROACHES TO SOILS' DECONTAMINATION FROM ANTHRAX' AGENT WITH ATTENTION TO UKRAINE NEEDS

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Summary. The Turkish experience on soil decontamination is based by modern conception of anthrax' driving forces – about clue role of bacteriophages in natural history of anthrax agent. This approach (as Kafkas University SOP) was proposed to approbation by Turkish-Ukrainian scientifically cooperation in framework of EU-project for soil decontamination.

Study was performed by the bacteriological (bacteria and phages isolation, identification, and cultivation, PCR) and epidemiological (surveillance with sampling, epidemiology analysis) methods. Samples were collected using basic methods from animal burial sites in Eastern Parts of Turkey (Kars, n=5) and Ukraine (Gorlovka, n=1). Soil desporulation conducted by germination of spores and elimination of vegetative *B. anthracis* with phages or peracetic acid. All procedures were standardized by SOPs of Kafkas University as in Turkish experience for soil desporulation.

The SOPs for surveys of the soil anthracic spores and phages, and soil desporulation (7 sites of sampling, 27 specimens) is effective, ecological friendly and economically low-cost. On two trials in Turkey (burial area "Külveren", S≈30m^2) and Ukraine (sample of soil from burial area "Gorlovka-2", weight 150 g) the contaminated soils (5×10^4 and 5×10^5, respectively) were de-sporulated during 24 hr. and hadn't residual viable spore in germinator presence even. Epidemiological analysis proves the Donbas region as most dangerous by anthrax' prognosis.

Turkish method is very useful to application in Ukraine, especially on the liberated territories of Donbas.

Keywords: spores of Bacillus anthracis, monitoring of soils contamination, decontamination procedure, Turkish technology, Ukrainian needs

Introduction. Anthrax to pose a dual threat – as a natural disease of all mammal species and as a biological weapon too. Therefore, attention to the problems of anthrax is growing in parallel to escalations of geopolitical situation – as such as aggressive Russian actions in Ukraine now. Over the last decade, by the efforts of research teams in the United States, Italy, Turkey and other countries, concept of anthrax' epidemiology has received important supplements and some details of this conception were even revised (Turnbull, 2002; Bouzianas, 2007; Schuch and Fischetti, 2009). To the last time, the lifecycle of *B. anthracis* often described by a short vegetative bursts in infected hosts alternating with long periods of dormancy as an environmental spore until disease is re-established. Environmental surveys show that *B. anthracis* can sporulate outside of anthrax carcasses, yielding an infectious cell type that is resistant to adverse conditions and is recoverable from the soil for long periods (Fig. 1a). To last time all events, which achieve of re-establishment of anthrax at contaminate territory, were some obscure and has a different explanations. Much more realistic and popular is Van Ness' paradigm (1971) about *B. anthracis* transformation from saprophyte into pathogen in so named "vegetative incubator area" (right part of Fig.1a). Modern dates of Rockefeller' Science Centre clear proof main factor of *B. anthracis* transformation from saprophyte into pathogen is lytic and lysogenic anthracic bacteriophages (Schuch and Fischetti, 2009). Other words, as like to "true" viruses, which are drivers of natural history of the highest forms of Earth Biota (like to animals and plants species by V. Vernadsky (1991), phages drive of behavior of the *B. anthracis* in environment. Therefore, modern view on the anthrax agent lifecycle (Schuch and Fischetti, 2009) stand the host-virus relationships at center of events of the *B. anthracis'* transformation from vegetative form to spores and back (Fig. 1b). Therefore, modern approaches to anthrax regulation may based

on promotions actions for rise of the "useful" anthracic bacteriophages activity in environmental objects of contaminate area. As on Fig. 1b show, such a "useful" phage may be ones that encoding of so-named σ factors: lysogenic *B. anthracis* with σ factor no able to sporulation (Mock and Fouet, 2001).

So to sanitation of environment especially important significance have the stimulation of phage's species that block the sporulation in mentioned vegetative incubator areas and in rhizosphere system (Fig. 1b). This principle is founded in base of decontaminate procedure that is developed in Kafkas University (t. Kars, Turkey). Turkish scientists revealed clue role of soil desporulation in its decontamination from *B. anthracis*. Therefore, regulation measures in "Turkish method" consist a both equivalent parts – 1) anthracic sporulation survey (sampling, studies of soil spores concentration, its life-ability, sporulation activity, soil phages characteristics); 2) anthracic sporulation control (provocation of soil spores growth, de-sporulation of soil by lytic phages, maintenance of soil phages and surveys quality of soil de-sporulation). Present paper summarized of initial results of "Turkish method" approbation in frame of Turkey-Ukraine scientific cooperation, which founding with European Union grant "AEDNet" (EU FP7).

Materials and methods. Bacterial & phages strains and growth conditions – The *B. anthracis* and phages strains used in this study were described in Table 1. As sources of environmental *B. anthracis* were soil of burial sites and pastures of the five agricultural holdings for cattle and/or sheep in Kars' Province (Turkey). In addition, we have taken two soil samples from old burial sites with cattle carcasses on territory of agricultural holding in Gorlovka district of Donetsk region (Ukraine) after explosions during war actions. All soil samples taken by standard "envelope" method – 5 points of the envelope at a distance of 1 m from its center point at a depth of 5–10 cm for each sample.

Bacterial cultures grown in Luria broth (LB) (Life Technologies, UK), brain-heart infusion broth (BHI) (Sigma-Aldrich, UK), or Leighton-Doi broth (Buyuk et al., 2013) according to standard protocols; plates were made by adding Bacto agar (Difco, US) to a final concentration of 1.6 %. All procedures with Turkish samples/strains conducted in Kafkas University, and with Ukrainian samples / strains – in NSC "IECVM".

B. anthracis spores isolation/titration method from soil- performed by schema above. Briefly, soil suspended

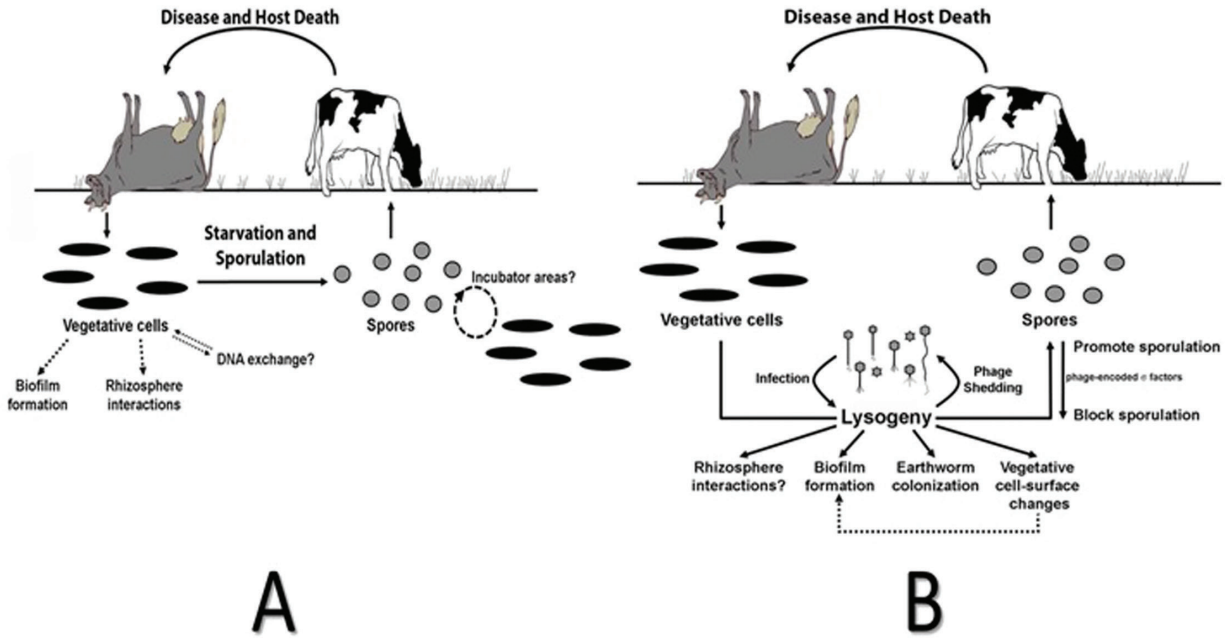
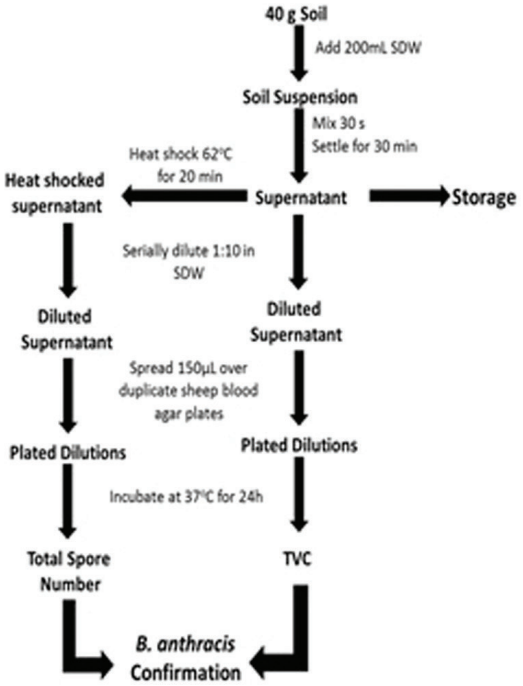


Figure – 1 The *B. anthracis* lifecycle by Schuch R1, Fischetti VA (2009) in our modification A – Former paradigm of *B. anthracis* lifecycle in which agent disease persists as a vegetative form in a model rhizosphere system, it's dormant spores (the infectious cell-type) are ingested by grazing hervibores and then germinate to produce a vegetative cell-type that causes fulminant disease in "Incubator area". B – Modern conception of *B. anthracis* lifecycle. Details – see the text



with sterile distilled water (SDW) and mixed well with shaking by hand. After incubation as drawing on schema, 2 ml of each supernatants used for isolation of both total viable cells and spores of *B. anthracis* (two portions, 1 ml of each). For bacterial cells and spores isolation were prepared 10 fold dilution on SDW. However, before bacterial spores isolation supernatant treated with heat at 62.5–63.0 °C for 15–20 min. and then dilution conducted.

All dilutions of sample was plated on blood agar plates in duplicate with a volume of 100–150 µl. Plates were incubated at 37 °C for overnight or 48 hr. at aerobic condition. Plates were checked for *B. anthracis* colony and performed the confirmation test.

Calculation of total viable spore (TVS) amount performed with considered of confirmed colonies of *B. anthracis* by next formulations:

$$\frac{\text{Colony number (confirmed as } B.\ anthracis)}{\text{Plated amount on agar plates X Dilution rate}} = \text{spore/ml}$$

The calculation method was improved with division of the spore amount in per milliliter to the value of soil amount (0,2 g) in 1 ml water to obtained total spore number in per gram soil.

Phage isolation method from soil – We used two different methods: 1 – for lytic phages isolation with a host without of inducers; 2 – total phage isolation with a host and Mitomycin C (Kyowa Hakko Kogyo Corporation, Tokyo, Japan). For lytic phage isolation, five grams of finely ground topsoil combined with 5 ml of Brain Heart Infusion (BHI) Broth (Sigma-Aldrich, US) and incubated at 37 °C for 2 hr. Followed this, 5 ml of a mid-log phase culture (the 3 to 5 hours culture of strain in BHI Broth with magnetic stirrer) of the Sterne strain of *B. anthracis* in BHI then added and the mixture incubated at 37 °C overnight. The following day top 5 ml of culture was harvested and filtered using a 0.22 µm membrane filter (Millipore, Massachusetts, US) to remove debris. The filtrate was stored at 4 °C until required. For total phage isolation, five grams of finely ground topsoil combined with 5 ml of Brain Heart Infusion (BHI) Broth and incubated at 37 °C for 2 hours. Then, 1 µl of 2 mg/ml solution of Mitomycin C added and the culture incubated for a further hour. Followed this, 5 ml of a mid log phase culture of the Sterne strain of *B. anthracis* in BHI was added and the mixture incubated at 37 °C overnight. The following day top 5 ml of culture was harvested and filtered using a 0.22 µm membrane filter to remove debris. The filtrate was stored at 4 °C until required.

Plague assay for phage activity detection – To determine the lytic activity of phage recovered using the method a plaque assay performed on Columbia Agar (CA) (Sigma-Aldrich, US) and *B. anthracis* Sterne used as host. The plaque assays carried out according to the method of T. G. Abshire (Abshire, Brown and Ezzell, 2005). Brief, host bacterial inoculum for phage propagation was prepared by transferring five to six isolated colonies from the CA culture grown overnight to 5 ml of sterile 10 mM phosphate-buffered saline (PBS), pH 7.2. CA plates were inoculated with 100 µl of the bacterial suspension, spreaded with a disposable plastic spreader until absorbed and 15 µl of the phage suspension delivered on agar surface. After fluids absorption, the plates were incubated at 37 °C overnight in inverted position. The cultures were inspected for plaque formation at regular intervals.

Phage amplification – To increase the concentration of *B. anthracis*-specific soil phages we mixed an equal volume of phage filtrate with a mid-log phase culture of the Sterne strain and incubated at 37 °C overnight. Next day the culture was spun down (8000 g, for 10 min), and supernatant filtered through 0.20 µm membrane filter. The phage titer was determined by performing a plaque assay. This process repeated until a sufficiently high-titer phage stock obtained.

Determination of the soil phages by routine test dilution (RTD) – The turbidity of an overnight culture of the Sterne strain of *B. anthracis* in BHI Broth was adjusted to a Mc Farland standard of 0.5 (1.5×10^8cfu/ml) using isotonic peptone-saline. The surface of a CA plate was then covered with 0.2 ml of this suspension and placed in a 37 °C incubator to dry for twenty minutes.

Ten microliters of each phage dilution was dropped onto the surface of the inoculated plates and left to dry for ten minutes. After which the plates were incubated at 37 °C and examined for the presence of plaques at 24-hour intervals. The most diluted suspension to produce complete clearing considered as the routine test dilution (RTD).

Soil desporulation procedures – These procedures were performed by two ways: 1 – on land plot that was positive on *B. anthracis* spore in Kars province; 2 – on contaminated soil sample from Gorlovka premise of Donbas region. Procedure for animal burial site in Kars province (Külveren) include the soil develop by desporulation mixture (special soil phages liquid culture in mix with *B. anthracis* germinator in standard concentration). The five soil samples were taken on land area of 1 m^2 by "envelope" method (see above) before and 24 hr. after soil developed by desporulation mixture. In second case the same desporulation mixture the same *B. anthracis* germinator in standard concentration implicated to Gorlovka' sample (5 g in three repetitions). Disinfection of vegetative forms of *B. anthracis* in Gorlovka sample conducted by peracetic acid with next neutralization of acid by Na-bisulfide [9].

Authors have considers all above procedures as Standard Operation Procedures (SOP) of Kafkas University (Turkey).

Results of study. Approbation of SOP for surveys of the soil anthracic spores and phages - In Table 1 the main results of soil anthracic spore and phages isolation and identification by Kafkas University' SOP is summarized. All soil samples contain both the *B. anthracis* spores and lytic phages. Levels of soil' contamination by spores depended from place of sampling. TVS-index of three probes (two from Turkey, one from Ukraine) was 5×10^5 and of another fours – 5×10^3 (one from Turkey, one from Ukraine), 5×10^4 and 5×10^7 (both from Turkey). *B. anthracis* bacteriophages soil-isolates showed titers < 10^{-1} RTD on first passages as on Sterne strain ("Turkish" isolates), as on "55" strain ("Ukrainian" isolates) – see numerator in RTD-column of Table 1. Nevertheless, on passages №№ 3–5 bacteriophages titers increased to 10^{-3}–10^{-5} RTD on Sterne strain ("Turkish" isolates), and to 10^{-2}–10^{-3} RTD on "55" strain ("Ukrainian" isolates) – see enumerators in RTD-column of Table 1. All of studied "Turkish" isolates had the capsules – main sign of *B. anthracis* pathogenicity.

In both Gorlovka' isolates capsule was absent, that we can explain by intensive using of live spore' and non-spore' vaccines against ruminant animal anthrax at this region of Ukraine.

On figures 2 and 3 the typical results of soil spores and phages growth and identification are presented. There are wide diversity of spore' bacteria species founding in all studied soil specimens (Fig. 2a). Main problem for this stage of work is right selection of colony with typical for *B. anthracis*' growth pattern: these are middle-size colonies with small tail and with surface with some yellowish

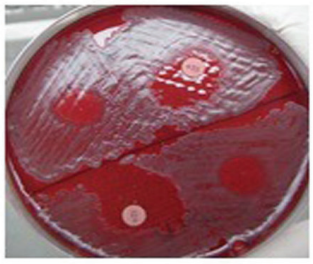
Table 1 – Results of primary analysis of soil' samples on presence of spores of the *B. anthracis*

Sample			Sites of sample	TVS	Capsula	RTD[1)]	PCR	
Code	Time of contamination	Time of sampling					PA	CAP
Dikme	2010	27.05.2014	Animal Burial	$5x10^5$	Pos.[2)]	10^{-5}	Pos.	Pos.
Selim 1	2006	27.05.2014	Animal Burial	$5x10^3$	Pos.	10^{-4}	Pos.	Pos.
Selim 2	2006	27.05.2014	Animal Burial	$5x10^7$	Pos.	10^{-3}	Pos.	Pos.
Külveren	2013	10.2013	Animal Burial	$5x10^4$	Pos.	10^{-3}	Pos.	Pos.
Subatan	2012	10.2013	Animal Burial	$5x10^5$	Pos.	10^{-4}	Pos.	Pos.
Gorlovka 1	U.N.[2)]	24.11.2014	Destroy area in Animal Burial	$5x10^3$	Neg.[2)]	10^{-2}	N.D.[2)]	N.D.
Gorlovka 2	U.N.	24.11.2014	Native area in Animal Burial	$5x10^5$	Neg.	10^{-4}	N.D.	N.D.

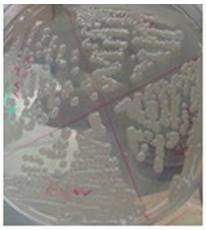
[1)]- on 3rd consecutive blind passage; in procedures with soil bacteriophages in Turkey used strain Sterne, in Ukraine - strain #55 (see text);
[2)]- U.N., un-known; Pos., positive result; Neg., negative result; N.D., not done

color. If done a right selection, on next stage of trial the bacterial colonies will growth that has sensitivity to penicillin and to *B. anthracis* gamma-phage (Fig. 2b). To *B. anthracis* pathogenic properties estimating very important to detect the capsule-formation activity of soil isolate. For this purpose, "Turkish experience " proposes the "Bicarbonat-agar" test (Fig. 2c) or PCR test with appropriate primers (not shown). Obligatory confirmation test on capsule-formation activity of soil isolates is routine develop of obtained agar cultures by any capsule staining method (Fig. 2d). In addition, for prognostic purposes, all agar cultures of soil *B. anthracis* are very important to examine of sporulation activity of originated from soil spores isolate by one of staining method (Fig. 2e). For this purpose Rakette staining is most suitable as simple and informative.

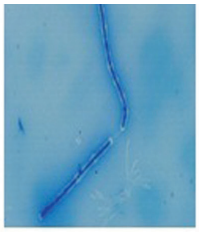
a - Soil anthracic and anthracoids spores' growth in blood agar



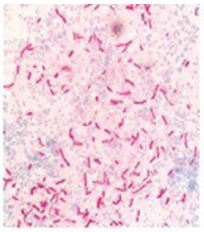
b - Confirmation test on soil-spores originated bacteria with penicillin-disk and by gamma-phage



c - Capsulated bacteria (shining colonies) from mixes of soil spores on "bicarbo-nate-agar" plate



d - Capsule staining (by Mc Fadyen) pa-ttern of soil' spore isolate Subatan of *B. anthracis*



e - Spore staining by Rakette method: pattern of soil' spore isolate Selim 1

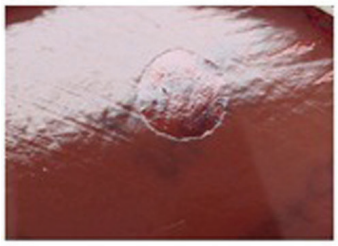
Figure – 2 Soil spores identification: routine methods' results

The specimens from different animal burial sites contained different spore concentrations – from 5×10^3 to 5×10^7 TVS. Even sampling in different places of the same animal burial site got the different results: in native place the quantity of soil' anthracis spores were much more (in 100 times) then in place of soil detonation (as explosion result, see Tab. 1 above).

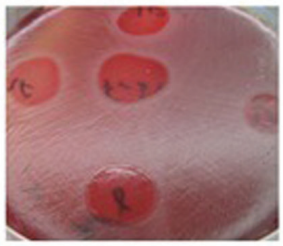
In part of soil phages' surveys, "Turkish experience" proposes the estimation of *B. anthracis* soil phages activity until and after growth on log-phase cultures of host (Fig. 3). As established by present study, the Sterne strain is the best choice of host culture (Fig. 3a, c, d); but the strain "55" is useful for preliminary investigations (Fig. 3b). Some hemolysis what we can see on Fig. 3b is caused by some anthracoid bacteria contamination of our phages isolate from Gorlovka specimens.

Soil sample № 3 (see table 1): partial lysis of Stern str. of the *B. anthracis* by phage isolate of 1st passage

Soil sample № 6 (see table 1): partial lysis of "55" str. of the *B. anthracis* by phage isolate of 1st passage



Soil sample № 3 (see table 1): full lysis of Stern str. of the *B. anthracis* by phage isolate of 3rd passage



Soil sample №№ 1–4: full lysis of Stern str. of the *B. anthracis* by phage isolate of 5th–7th passages

Figure 3 – Soil phages of *B. anthracis* growth by sequential passages in host agar-cultures: routine methods' results

Approbation of SOP through trials on soil desporulation – Trials with soil desporulation by bacteriophage-containing cocktail were effective at only environmental temperatures ≥ 16 °C (n=5). Low atmospheric temperatures (lower than 16 °C) did not provide adequate of *B. anthracis* spores germination and lytic bacteriophage activity. For successful desporulation of soil the critical significance had the level of lytic activity of the bacteriophage which used in of mentioned cocktails. As we can't achieve of lytic bacteriophage activity to ≥ 10^9 RTD in Ukraine, our attempts to use of Gorlovka' phage isolate to soil samples desporulation were partly unsuccessful: residual spore' contamination (≤ $10^{2.5}$CFU)

in soil sample dilution 10^{-2} and even 10^{-3} (in untreated sample – 10^{-5}) was registered. So for soil samples desporulation we used peracetic acid instead phage at last stages of soil desporulation. In this case additional advantage was in efficiency of peracetic acid for at low temperatures unlike to phage.

A main result of desporulation trials is summarized in Table 2 and show on figure 4.

Table 2 – The results of trials for soil anthracis desporulation

Desporulation areas					Total Viable Spores				
Code	Time of contamination	Scope of work	Time of processing	Processing method[1)]	Before	After[2)] 24hr	7 d	14 d	21 d
Külveren (Animal Burial Area)	2013	≈ 30 m²	XI-2014	A	$5x10^4$	Neg.	Neg.	N.D.	N.D.
Gorlovka 2 (Sample from Animal Burial Area)	U.N.	150 g	XII-2014	B	$5x10^5$	Neg.	Neg.	Neg.	Neg.

[1)]- A, germination and bacteriophagal lysis simultaneously; B, germination, than disinfection with peracetic acid (see text);
[2)]- Neg., negative result; N.D., not done

a – Soil spores growth control in dilution of sample 10^{-1}. Soil sample taken just after desporulation mixt application

b – Soil spores growth control in dilution of sample 10^{-2}. Soil sample taken just after desporulation mixt application

c – Soil spores growth control in dilution of sample 10^{-2}. Soil sample taken 24 hr. after desporulation mixt application

Figure 4 – Quality control of desporulation procedures (see text for details)

In both cases the similar results were obtained – application of Turkish technology allowed to full omit the *B. anthracis* spore from Külveren' animal burial area (Turkey) and from sample of soil from Gorlovka' animal burial area in (native part of area) during 24 hr. after soil processing. Absence of anthracis spores in processed objects registered in all period of quality control of trials on the SOP for decontamination: 7 days in Külveren' animal burial area (Turkey) and 21 days for sample of soil from Gorlovka' animal burial area, which exposed under 18 °C in presence of spore germinator during 24 hr. before the last examination date (21 day apart from processing start).

Analyzing the previous literature described and data above, it is possible to make the solution that Turkish method for soil desporulation in based on modern scientific conception and allow to soil release of anthrax spores and bacteria. Therefore this method is very useful for Ukrainian needs as effective, ecological friendly and economically low-cost.

On figure 5 shows the cumulative dates on anthrax outbreaks (all animal species) in Ukraine for 92-years period (from 1920 to September 2012, by Yanenko et al. (2013) with modification). As Prof. Dr. Sc. Zaviruha A.I. claims the main threats from anthrax in Ukraine are originated from hurry sites of modern and especially ancient animal burial area (Zaviriuha, Slupskaya and Yavorskaya, 2014). Agricultural processing of soil, other land uses or especially soil detonations through explosions during war actions contribute to ejection of dormant spores of burial sites on the surface of the earth. So, as we can see on animal anthrax incidence in Donbas region (see Lugansk & Donetsk oblast on map),

where arithmetic average rate is closer to the highest level in Ukraine (1046 cases for 92 years, Fig. 5), we can conclude that this region of war is most threatening about anthrax outbreaks in the nearest future. These threats increase many times because of the many possibility of terrorist use of the causative agent of anthrax – and not only in Donbas area.

Therefore we have many reasons to continuation of development and use of Turkish method for soil desporulation in Ukraine.

Conclusion. The studies based on the combination of phages and spore germinants prove the effectiveness of Turkish method for soil desporulation and show highly promising for implementation in Ukraine. And still better,Turkish scientists are ready for sharing their experiences with Ukranian scientists and cooperation.

Acknowledgements. The authors would like to thank Prof Les Baillie from Welsh School of Pharmacy, Cardiff University, UK for his scientific contributions.

Figure 5 – Map of anthrax outbreaks on all animal species for 92-years period. Donbas area marked by a dotted black (see text for detail)

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