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TOXIGENCE OF ANTHRAX VACCINE STRAINS

G. A. ZAVIRIYHA, *Candidate of Agricultural Sciences*
https://orcid.org/0000-0002-46028477
"State Center for Innovative Biotechnology", Kyiv, Ukraine
E-mail: annazavir@gmail.com
U. N. YANENKO, *Candidate of Veterinary Sciences*
https://orcid.org/ 0000-0001-5678-3356
"State Center for Innovative Biotechnology", Kyiv, Ukraine
E-mail: ulyanakuzyk@ukr.net,
N. I. KOSYANCHUK, *Candidate of Veterinary Sciences, Associate Professor*
Department of Veterinary Hygiene Named After Professor A. K. Skorokhodko
https://orcid.org/ 0000-0002- 3055-8107
National University of Life and Environmental Sciences of Ukraine, Kyiv, Ukraine
E-mail: ninaiva2@ukr.net

Abstract. *The article presents the results of the studying of anthrax strains and Bacillus anthracis-like strains on the formation of toxins. We found that anthrax vaccine strains actively produce exotoxins to the culture fluid. The amount of specific protein is different under the same incubation conditions and depends on the individual characteristics of the microorganism population, because of this, different titers of the toxin are registered.*

Strain B. anthracis K-79 Z (vaccine) with the same number of planting microbial cells and growing on the same culture medium and at the same temperature produces by two orders of magnitude more exotoxin than strains B. anthracis Tsenkovsky II IBM 92Z (virulent), B. anthracis Stern 34F2, (vaccine), B. anthracis 55 (vaccine), B. anthracis SB (vaccine), B. anthracis Tsenkovsky I (vaccine, apathogenic). The amount of exotoxin may change if the pH of the medium changes. The activity of exotoxin production, when the pH changes, depends on the characteristics of the anthrax strain.

Keywords: *anthrax, exotoxin, exotoxin production*

Introduction

Anthrax is a part of the most dangerous infectious diseases common to humans and animals. This disease is common in all mammals, most often among cattle (large and small) and horses. The causative agent is the gram-positive bacterium *Bacillus anthracis*, which forms spores. The infectious dose of the pathogen is only 1–10 spores. They fall victim to the blood of a sick animal or a corpse. The pathogen forms spores that, once in the soil, can persist for more than 100 years. After reaching a favourable environment, the spores become active in 1–6 days, some spores may intensify after 60 days or more (Cizauskas et al.,

2014; Hendricks et al., 2014; Shadomy et al., 2016; Chugh, 2019).

For this reason, the area contaminated with anthrax spores is a factor in the transmission of the disease indefinitely.

During its stay in the soil, the anthrax pathogen periodically restores its population, thus maintaining the infectivity of the territory. To reproduce its population and mass, *B. anthracis* infects animals or humans, and with their corpses, it re-enters the soil and closes the natural cycle of its biology.

Humans can become infected by anthrax via respiratory system (inhalation), gastrointestinal tract, and through the skin (this is the most common form and 95% manifests as a malignant pustule). The inhaled form of anthrax is the most severe, as it often causes septicemia and meningitis with high mortality and the highest risk of man-made spread. The gastrointestinal form of anthrax occurs when consuming meat and meat products of poor quality (Glomski et al., 2007; Hashemi et al., 2015; Owen et al., 2015; Vieira et al., 2017; Mwakapeje et al., 2018).

Between 2,000 and 100,000 cases of anthrax are reported worldwide each year. This disease remains a major health threat in Africa, the Middle East, South America, Central Asia, and Haiti (Doğanay & Eşel, 2008; Abboud et al., 2009; Liu et al., 2020). Anthrax spores can be used as a target for bioterrorism. In 2001, spores of the pathogen were mailed to exceptional individuals in the United States, which caused 22 cases of cutaneous, inhalation, and meningeal anthrax and 5 deaths (Abrol, 2016; Walsh et al., 2018).

Stability of spores for many years, unpretentiousness to the conditions of cultivation leads to human disease or death – all this makes anthrax a primary pathogen of the NIAID category. The pathogen causes 45–90% of mortality even with timely treatment with antimicrobial drugs (Mourez, 2004). High mortality is associated with the rapid development of bacterial infection and the action of tripartite toxin (Crowe et al., 2010).

Both field and vaccine strains of the anthrax pathogen have the ability to produce toxins. *B. anthracis* has two main virulence factors: poly-γ-D-glutamic acid capsule and tripartite toxin (Mock & Fouet, 2001). The capsule plays an antiphagocytic role and allows bacteria to avoid absorption by macrophages (Zwartouw & Smith, 1956). The tripartite toxin contains protective antigen, lethal factor, and oedema factor (Stanley et al., 1960: Stanley et al., 1961). These toxins act on the innate and adaptive immune system and affect the subsequent consequences of the disease (Tournier et al., 2009; Moayeri et al., 2015). Field and vaccine strains clearly differ in the lethality and the amount of toxin production. Field strains produce little toxin during *in vitro* cultivation, but they are more aggressive (predominate lethal and oedematous fractions). Vaccine strains produce more toxin, which is dominated by a protective fraction.

All anthrax vaccines develop antitoxic immunity in a vaccinated animal, but the ability of anthrax microbes to produce an exotoxin is not the same. The stress and duration of immunity (immune protection of vaccinated animals) depend on this property, so studies of the toxigenicity of anthrax strains are relevant.

Analysis of recent sources and publications

Exotoxins of the anthrax pathogen as biotechnological objects are relevant for many scientists in different countries (Friebe et al., 2016).

Studying the protective factor of the antigen and the mechanism of formation have shown that the protective factor may be a new means for controlling cytotoxicity (Kintzer et al., 2009) and studying the complex action of exotoxins as toxic complexes (Lacy et al., 2002). Studying the mechanism of action of a protective exotoxin on a cell against the background of the lethal and oedematous toxin are relevant (Chaudhary et al., 2012; Slater et al., 2013). The use of protective antigen of the anthrax pathogen as an inhibitor of breast cancer cell development has shown the prospects for the development and application of new therapeutic approaches in the fight against tumours (Felix et al., 2020).

Particular attention is paid to the studying of the epizootic situation and disease monitoring worldwide. Creating a unified surveillance system, strengthening cross-sectoral coordination on prevention and control, biological and social strategies for anthrax prevention focuses scientists' attention on endemic regions (Sahoo et al., 2020). Improving anthrax vaccines for humans and animals is an important stage in preventing infection. We are conducting research to find new approaches to the use of antibiotics in the schemes of anthrax vaccines. There is a question of disposable vaccine creation (Clark & Wolfe, 2020).

The development of vaccines, which preserve natural antigenic structures that mimic natural infection, and the studying of the effects of antibodies that neutralize toxins are an important research area (Dumas et al., 2020).

The purpose is to study the properties of toxins formation by anthrax pathogens and *Bacillus anthracis-like* strains.

Material and methods of research

To conduct the experiment we took lyophilized museum cultures of anthrax vaccine strains and *Bacillus anthracis-like* strains: *B. anthracis* Tsenkovsky II IBM 92 Z (virulent), *B. anthracis* K-79 Z, *B. anthracis* Sterne *34F2*, *B. Anthracis* 55, *B. anthracis* SB, *B. anthracis* Tsenkovsky I, *B. anthracoides* 67, *B. Subtilis* 17, *B. cereus* 8035 (virulent), which are stored in collection of microorganisms in the "State Center for Innovative Biotechnology". The methods for studying vaccine strains described by S. G. Kolesov, generally accepted in microbiological practice, were used (1976).

The agglutination reaction and the concentration of the spore suspension for each strain were determined in colony forming units (CFU) by scientific and methodological recommendations "Laboratory diagnosis of anthrax in animals, detection of pathogens from pathological and biological material, raw materials of animal origin and environmental objects" (Skrypnyk et al., 2015). Statistical processing of the obtained research results was performed using the program "Microsoft Excel 2011" for Windows.

The titer of microorganisms toxigenicity in the causative agent of anthrax and *Bacillus anthracis-like* strains was determined using the disc precipitation reaction (DPR) by the method improved by A. I. Zaviryukha (Zaviryukha & Stepanyuk, 1978). Precipitated anthrax serum RP № 3905-14-0403-08, manufactured by Kherson State Biological Factory, was used for the study.

Bacterial cells of vaccine strains and *Bacillus anthracis-like* strains were grown on standard culture media: broth and meat-peptone agar, and injection of 5 and 10 % gelatine at pH 7.2 – 7.6 and a temperature of 37.0 ± 1.0^0 C.

From microbial colonies grown on agar, smears were prepared and stained according to Gram, Mikhin, Olt, Rebiger, Romanowsky-Giemsa, the length and width of microbial cells were measured with a micrometre, and their shape and the presence of spores were determined. The shape and physical characteristics of the colony were studied.

Antigens were prepared by autoclaving from the microbial mass of each strain, which was investigated in the Ascoli reaction and in the DPR with anthrax serum for precipitation. We prepared antigens from the culture fluid of vaccine strains, which were filtered through a bacterial filter and examined by the DPR and Ascoli.

The control was antigens made from bacterial mass and sterile filtrate of culture fluids of anthracoid microorganisms *B. Anthracoides* 67, *B. Subtilis* 17, *B. cereus* 8035 (virulent).

Results of the research and their discussion

Investigated extracellular anthrax toxins, which were obtained by the method mentioned above. The results of the examination by the reaction of Ascoli and the DPR are presented in Table 1.

Our data indicate that the virulent strain *B. anthracis* Tsenkovsky II IBM 92Z and the avirulent *B. anthracis* K-79Z produce extracellular toxin outside the cell membrane in a liquid nutrient medium. The toxin reacts with antibodies of the precipitating anthrax serum in the precipitation reaction. However, the amount of exotoxin in the culture fluid of virulent and avirulent strains is not the same. We noted the ability to produce exotoxin at a titer of 1:32 in vaccine strains of anthrax.

1. The results of studying the sterile filtrates of culture fluids of microbes of the genus *Bacillus*, which were examined by the Ascoli reaction and the disk precipitation reaction

Strain	Ascoli								DPR							
	dilution of culture fluid															
	n	1:2	1:4	1:8	1:1	1:3	1:6	1:128	n	1:2	1:4	1:8	1:16	1:3	1:6	1:128
B. anthracis K-79 Z	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. anthracis Tsenkovsky II IBM-92 Z	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-
B. anthracis Sterne 34F2	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-	-
B. anthracis 55	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-	-
B. anthracis SB	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-	-
B. anthracis Tsenkovsky I	+	+	+	+	+	-	-	-	+	+	+	+	+	-	-	-
B. cereus 8035	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. anthracoides 67	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-
B. subtilis 17	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-

Note: n – native fluid; + positive reaction; – negative reaction.

Saprophytic spore-forming bacilli of *B. anthracoides* 67, *B. subtilis* 17, *B. cereus* 8035, and *B. subtilis* 17 do not produce a specific toxin, and therefore their filtrates of culture fluids do not react by precipitation with antibodies of precipitated anthrax serum.

We investigated the impact of pH on the production of exotoxin by anthrax strains, which began with the production of bacterial masses with an identical number of microbial cells – 30.2–30.8 × 10^6 CFU.

Colonies that grown on agar were seeded in a liquid nutrient medium – meat broth with different pH values – 6.5; 8.5; 7.5. They were incubated in a thermostat for 48 hours at 37^0 C. Then we filtered the bacterial mass through bacterial filters type F5. The received filtrates were checked according to DSTU 4483:2005.

We performed the DPR with culture fluid filtrates to determine the anthrax protein titer. The results of the examination are presented in Table 2.

2. Effect of pH on exotoxin production by anthrax vaccine strains, M ± m, n = 10

№	Strain	Number of grown microbial cells, CFU	pH of the medium	Exotoxin titer in the DPR
1.	B. anthracis K-79 Z	30.5 × 106	6.5	8
			7.5	64
			8.5	32
			M ± m	34.66 ± 16.22**
2.	B. anthracis Tsenkovsky II IBM 92 Z	30.7 × 106	6.5	4
			7.5	8
			8.5	4
			M ± m	5.33 ± 1.33**
3.	B. anthracis Sterne 34F2	30.2 × 106	6.5	8
			7.5	32
			8.5	16
			M ± m	18.66 ± 7.05**
4.	B. anthracis 55	30.8 × 106	6.5	8
			7.5	32
			8.5	16
			M ± m	18.66 ± 7.05**
5.	B. anthracis SB	30.5 × 106	6.5	8
			7.5	32
			8.5	16
			M ± m	18.667 ± 7.05**
6.	B. anthracis Tsenkovsky I	30.2 × 106	6.5	8
			7.5	16
			8.5	8
			M ± m	10.66 ± 2.66**

Note: * P < 0.05; ** P < 0.02; *** P < 0.01; **** P < 0.001.

Indicators of Table 2 show that at different pH of the medium, the amount of specific protein in the strains increased differently (1:8–1:64 – *B anthracis* K-79Z, 1:8–1:32 – *B. anthracis* Sterne 34F2, *B. anthracis* 55, and *B.* anthracis SB, 1:8–1:16 – *B. anthracis* Tsenkovsky I*)*. At the same cultivation conditions, a high titer of specific anthrax protein showed the exotoxin *B. anthracis* K-79Z – 1:64 at pH 7.5. Strains *B. anthracis* Sterne 34F2, *B. anthracis* 55, and *B. anthracis* SB at pH 7.5 produced exotoxin at the same level, and it was 1:32. Also, in these strains, the same amount of exotoxin was observed when cultured in a medium at a pH of 6.5, which was 1:8. When the pH was raised to 8.5, the exotoxin purge of these strains was at 1:16. This indicates similar characteristics of these vaccine strains and the effect of pH on the production of exotoxin.

Conclusions and future perspectives of the study

Vaccine strains of anthrax actively produce exotoxins in the culture fluid. The amount of specific protein under the same incubation conditions is different and depends on the individual characteristics of the microorganism populations.

The *B. anthracis* K-79 Z strain with the same number of microbial cells and under cultivation in the same culture medium and temperature conditions produces by two orders of magnitude more exotoxin than *B. anthracis* Tsenkovsky II IBM 92Z, *B. anthracis* Sterne 34F2, *B. anthracis* 55, *B. anthracis* SB and *B. anthracis* Tsenkovsky I.

Vaccine strains of anthrax, which we studied, produce exotoxin when the pH of the medium. Its amount varies depending on the pH of the medium. The activity of exotoxin production when the pH changes depends on the characteristics of the anthrax strain.

The Perspective: we plan to continue the studying of the toxigenic properties of anthrax strains in order to obtain an exotoxin that will be used in the development of new drugs against anthrax in humans and animals.

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Анотація. *У статті представлені результати дослідження штамів сибірки та антракоїдів щодо утворення токсинів. Ми виявили, що вакцинні штами сибірки активно продукують екзотоксини в культуральну рідину. Кількість специфічного білка різна за однакових умов інкубації й залежить від індивідуальних особливостей популяції мікроорганізмів, через що реєструються різні титри токсину.*

Штам B. anthracis K-79Z (вакцина) з однаковою кількістю посіяних мікробних клітин і за вирощування на одному й тому ж поживиному середовищі та за однакової температури виробляє на два порядки більше екзотоксину, ніж штами B. anthracis Tsenkovsky II IBM 92Z (вірулентний), B. anthracis Sterne 34F2, (вакцина), B. anthracis 55 (вакцина), B. anthracis SB (вакцина), B. anthracis Tsenkovsky I (вакцина, патогенна). Кількість екзотоксину може змінюватися внаслідок зміни pH середовища. Активність продукування екзотоксину внаслідок зміни pH середовища залежить від характеристик штаму сибірки.

Ключові слова: *сибірка, екзотоксин, продукція екзотоксину*