



Features of exotoxin production of vaccine strains of anthrax pathogen for use in the veterinary industry

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Abstract. Exotoxins that produce vaccine strains of the anthrax pathogen are the main source of immunogenicity of anti-selective vaccines used in veterinary medicine. The relevance of the study is due to the search for the most suitable vaccine strains of the anthrax pathogen to obtain

Suggested Citation:

Zaviryukha, G., Vyshnytska, I., Yanenko, U., Sorokina, N., & Vasylieva, T. (2024). Features of exotoxin production of vaccine strains of anthrax pathogen for use in the veterinary industry. *Ukrainian Journal of Veterinary Sciences*, 15(1), 84-103. doi: 10.31548/veterinary1.2024.84.

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high production of exotoxin as a factor of the effectiveness of drugs for the implementation of preventive and safety measures in the field of veterinary medicine. In this regard, the purpose of the study was to examine the productive properties of microbes of the *Bacillus* genus regarding the production of exotoxin under changes in cultivation conditions during incubation. Microbiological and biotechnological methods and comparative statistical analysis are used to examine vaccine strains of the anthrax pathogen. Strains are selected according to the intensity of growth on nutrient media. A biotechnological approach to obtaining a specific anthrax protein is used to analyse the production of exotoxin by vaccine strains of the anthrax pathogen. When cultured on identical nutrient media, the vaccine strains produce different amounts of exotoxin. Virulent (*B. anthracis* IBM-92 Z), vaccine (*B. anthracis* K-79 Z, *B. anthracis* Sterne 34F 2, *B. anthracis* 55, *B. anthracis* SB, *B. anthracis* Tsenkovsky II) strains, and anthrax cultures (*B. cereus* 8035, *B. anthracoides* 67, *B. subtilis* BKM 17) are examined. In the course of experimental work, it is determined that the production of exotoxin of various anthrax pathogen strains depends on the medium's pH. It is established that with identical pH values of the medium and cultivation conditions, the highest production of exotoxin was shown by the vaccine strain *B. anthracis* K-79 Z. The titer of a specific anthrax protein was 1:64. Changes in the pH of the medium during the cultivation of strains affect the amount of exotoxin formation – the main factor in the formation of specific immunity against the anthrax pathogen. The results of the study can be applied by specialists of the veterinary service to select antigen producers in the development of new drugs against anthrax in animals based on exotoxins

Keywords: anthrax; toxin formation; cultivation; medium pH; virulent strains; reference cultures

Introduction

Anthrax is an infectious disease caused by a spore-forming gram-positive, rod-shaped bacterium, *Bacillus anthracis*. In the large *Bacillus* genus, *B. anthracis* is the only obligate pathogen. As noted by M. Omodo *et al.* (2023), anthrax is a zoonotic disease transmitted through the soil by a pathogen found on all continents of the globe. Anthrax is potentially contagious to most mammals. However, first of all, it affects ruminants. This bacterium survives for decades as spores in meadows contaminated with abandoned or buried corpses of animals that died from anthrax. R. Liddington (2021) draws attention to the fact that people become infected with anthrax through contact with infected animals or by eating contaminated animal products. In 95% of cases, when a person is infected with anthrax, the skin form is most common. E. Kisaakye *et al.* (2020) indicate that

WHO estimates that between 2,000 and 20,000 human cases of anthrax are reported annually in the world.

Infection with *B. anthracis* is possible through the skin, gastrointestinal, or respiratory tract. Anthrax exists in two forms: vegetative cells (inside the host) and endospores in the soil or environment, where, as noted by S. Topluoglu *et al.* (2021), it can remain viable for decades to come. The global distribution of anthrax, in most cases, is determined by the presence of soils that support the survival of spores, namely those that are rich in organic substances and calcium and have a pH > 6.4. W. Ashenefe *et al.* (2022) proved that a warming climate will increase the risk of anthrax in some regions of the world. The preservation of spores and unpretentiousness to the conditions of existence created prerequisites for the potential

use of bacteria as a weapon for bioterrorism. As stated by E.K. Dumas *et al.* (2020), anthrax outbreaks and isolated cases of animal and human infection have been reported in many countries. High mortality in the invasive form of anthrax and the development of shock during the disease are quite common consequences of this disease compared to the effect of other bacteria on the body. Therefore, developed countries pay considerable attention to this deadly infection.

Bacillus anthracis, the etiological agent of anthrax, becomes particularly virulent due to the capsule and two AB-type toxins: lethal factor LF and edematous factor EF. These toxins primarily disable immunocompetent cells. Both toxins are transferred to the host cell via the adhesin-Internalin subunit, a protective PA antigen. T.G. Sumithra *et al.* (2021) report that PA allows LF to reach intraluminal vesicles, where it remains active for a long time.

The main criterion for the development of modern anti-anthrax agents in veterinary medicine is the use of anthrax vaccine strains. According to W. Liu & E.M. Nestorovich (2021), it follows that the feature of *B. anthracis* is a property to produce exotoxin outside its cell. This product of metabolism of the vegetative form of bacilli consists of three fractions: protective, edematous, and lethal. The ability of anthrax microbes to produce exotoxin varies. All anthrax vaccines form antitoxic immunity in the vaccinated animal. The intensity and duration of immunity depends on this property, so the examination of toxigenicity of anthrax strains is relevant.

The toxin contains various combinations of protective antigen (PA), lethal factor (LF), and edema factor (EF). These three proteins combine to form two toxins: a lethal toxin (LT, a combination of PA and LF) and an edematous toxin (ET, a combination of PA and EF). Toxins alter the signalling pathways of host cells to interfere with the innate immune response in the early stages of infection and cause a

vascular collapse in the later stages of the disease. According to R. Liddington (2021), toxins not only affect the innate and adaptive immune system but also determine the subsequent consequences of the disease. Due to the key role of the protective antigen (PA) in the action of anthrax toxin, efforts are being made to develop new vaccines consisting of a purified recombinant form of PA (rPA) adsorbed on an aluminium adjuvant that can cause a persistent neutralising effect. PA is immunogenic and causes the formation of toxin-neutralising antibodies that correlate with anthrax protection. Vaccines against toxin-mediated bacterial diseases such as diphtheria, tetanus, and anthrax protect by triggering sustained antibody responses that neutralise the toxin. However, attempts to develop such vaccines are difficult due to the lack of stability of the vaccine during storage.

When cultivating field virulent strains of *B. anthracis* in the laboratory, a slight production of the toxin is recorded, but it has a high aggressiveness. In the composition of the toxin of the field strain, the lethal and edematous fractions occupy more than 70%. Thus, as proved by W. Liu & E.M. Nestorovich (2021), the protective fraction accounts for less than 30%. Anthrax vaccine strains produce exotoxin, which is dominated by the protective fraction. When grown under identical conditions, they produce different amounts of exotoxin. Saprophytic spore-forming aerobic microbes from the *Bacillus* genus produce their own metabolites in the culture fluid but do not form a specific anthrax toxin. The ability of a vaccine strain to produce exotoxin *in vitro* depends on many factors: genetic makings, composition and type of nutrient medium, pH values, duration of cultivation, temperature, and number of cultivation passages. M. Manish *et al.* (2020) note that an important role in the creation of drugs using anthrax is played by the selection of anthrax vaccine strains.

From the above, the purpose of the study is to investigate the ability of various microbes of the *Bacillus* genus to produce exotoxins outside the cell wall when culture conditions change during incubation in a veterinary laboratory. Therewith, special attention was paid to the effect of the pH of the medium on the production of exotoxin, which is an important stage in the toxin formation and further use of anthrax pathogen strains in the case of creating new veterinary drugs. The main development of drugs based on anthrax exotoxins was carried out during the lifetime of Academician A.I. Zaviryukha, under his leadership a group of scientists (DNU State Center for Innovative Biotechnologies, Ukraine, Kyiv). This study is a continuation of scientific work on the development of modern technologies for obtaining exotoxins of pathogenic microorganisms.

Literature Review

K.A. Simonsen & K. Chatterjee (2019) indicated that anthrax actors, once in a favourable environment, during reproduction in a living organism (*in vivo*) and on liquid nutrient media (*in vitro*), germinate quickly and secrete exotoxin outside the cell wall. Anthrax toxin consists of three proteins: protective antigen (PA, 83 kDa), lethal factor (LF, 90 kDa), and edema factor (EF, 89 kDa). These three polypeptides are encoded by the pXO1 plasmid and form dicyclic combinations. The combination of LF and PA forms a lethal toxin (LT), whereas EF, in combination with PA, creates an edematous toxin (ET). Two protein components of anthrax toxin (EF and PA) increase the host's susceptibility to infection by inhibiting PMN function and increasing the body's resistance. The researchers proved that the combination of LT and ET is a disease factor. Instead, separately, the three toxin components (PA, LF, and EF) are non-toxic, which follows from the mortality of mice with LT and ET.

According to the results of the study, today, there is a wide possibility of using attenuated toxins as drug delivery systems. J.L. Dale *et al.* (2018) noted that experimental preparations containing exotoxins had high preventive properties. PA was shown to be a harmless toxin subunit that triggers a protective immune response and is the basis for all preventive measures against anthrax. According to J. Frydrych *et al.* (2018), M. Manish *et al.* (2020) and J. Tournier & C. Rougeaux (2020), anthrax protein vaccines licensed for human use include partially purified PA enhanced with various excipients.

In recent years, anthrax toxin has been modernised, and it acts as a targeted anti-angiogenic antitumour agent that kills tumours and has excellent therapeutic performance in experiments on animal models. Two conceptually different functions were added to PA to achieve high specificity when targeting the tumour. Anthrax toxin is a powerful tool for delivering proteins and drugs to cells. The ability of PA to various modifications and the growing number of new LF drug conjugates, as proved by C.J. Young *et al.* (2018) and E.S. Fischer *et al.* (2019), make this system a fairly versatile tool not only for antitumour therapy but also for use in other areas of veterinary medicine and biomedicine. J.M. Fonseca *et al.* (2020) indicate that anthrax toxin has proven to be a universal system for delivering drugs to cells from multiple enzyme fragments. This highly efficient delivery system was further modified by introducing ubiquitin as a cytosolic cleavage site into lethal fusion proteins. Such developments are conducted for the purpose of targeting tumours and delivering drugs to them.

Anthrax toxin receptors: tumour endothelial marker 8 (TEM8) and capillary morphogenesis gene 2 (CMG2) are endothelial receptors involved in extracellular matrix homeostasis and angiogenesis that are selectively activated in many tumours. One of the methods of

influencing these receptors is the protective antigen (PA), a protein produced by *B. anthracis*. T. Crawford *et al.* (2019) specified that toxins targeting PA selectively inhibit tumour growth and angiogenesis, but the selectivity of PA for the tumour is still being investigated. Application of *B. anthracis* strains with reduced protease was identified to be appropriate in combination with drugs to prevent the trigger of an immune response at the stage of cancer treatment. M.A. Walker *et al.* (2020) used protective antigen (PA) for specific targeting of different types of tumours.

D. Diaz-Arévalo *et al.* (2021) proved that the involvement of anthrax exotoxin in needle-free mucosal vaccines has positive results. Therewith, the immunogenic properties of anthrax exotoxin, which was used to develop combined drugs, were examined. It is noted that during the development of an influenza vaccine, its composition should be reviewed annually due to antigenic changes in circulating strains of the influenza virus. Due to seasonal drift and changes in circulating strains, the flu vaccine does not always correspond to circulating strains, and the adjuvants included in it are insufficient to induce a protective effect on long-lived memory cells. Adjuvants play an important role in the immune response to the vaccine and *Bacillus anthracis* the detoxified anthrax edema toxin, which consists of the protective antigen PA and the N-fragment of edema factor (EFn), showed an improved effect on humoral and cellular immune responses. As a result, a universal influenza vaccine design was developed, consisting of three tandem repeats of influenza antigen M2E plus HA2 and detoxified toxin EFn, which is associated with the PA component, and methods used to confirm protection. This will help develop a vaccination strategy using detoxified anthrax toxin for intranasal delivery of influenza antigen.

Despite the effectiveness of modern vaccines, scientists are searching for the development of the most areactogenic drugs, namely, improving anti-selective vaccines to reduce irritation and inflammation at the injection site. Similar reactions, as noted by S. Alameh *et al.* (2020) and T.G. Sumithra *et al.* (2021), occur when exposed to edematous and fatal factors. M.A. Smiley *et al.* (2019), D. Żakowska *et al.* (2019), and D.R. Weilhammer *et al.* (2020) proved that anthrax vaccines based on mutant variants of the exotoxin PA have less toxicity and greater immunogenicity. PA is a central component of anthrax toxin, which plays a leading role in protecting against encapsulated and non-encapsulated anthrax strains. Vaccines based on subunit rPA have a good safety and protection profile. However, there are problems of PA instability that are substantially aggravated by the use of aluminium adjuvants. New adjuvant compositions, dry preparations, and mutant forms of PA that are resistant to proteolysis and deamidation can solve this problem. Therefore, as stated by O.A. Kondakova *et al.* (2019), the development of a modern anthrax vaccine requires further research.

The protective antigen is the main protective immunogen in anthrax vaccines licensed in the United Kingdom and the United States. Repeated administration of vaccines is required to protect the body from this disease. However, depending on how they are developed, vaccines are relatively coarse products containing trace amounts of LF, EF, and other bacterial antigens that contribute to reactogenicity. LF and its individual domains have been confirmed to stimulate a protective antibody response in animals and humans. Combining protective regions into a single fusion protein is a more efficient, cost-effective, and practical approach. A fusion protein containing the N-terminal PA-binding domain LF (LFn) and the C-terminal PA-binding domain of the host cell can protect mice

from lethal anthrax infection. T.B. Gallagher *et al.* (2019) proved that the production of a single vaccine containing protective regions against LF and PA is cost-effective and can provide a wider range of protection than a vaccine containing only PA.

P. Pilo & J. Frey (2018) and V. Savransky *et al.* (2019) conducted a number of studies on the development of anthrax vaccine with recombinant protective antigen (rPA) (rPA7909), which is intended for post-exposure prevention of diseases caused by suspicious or confirmed *Bacillus anthracis*. The reaction at the injection site in animals treated with the control adjuvant is similar to that in animals treated with rPA7909 for inflammation. The inflammatory response at the injection site and drained lymph nodes was consistent with the expected immune stimulation and indicated a favourable safety profile for rPA7909. Although anthrax is rare, this microorganism can potentially be used in bioterrorism because it produces endospores that can withstand harsh environmental conditions. Therefore, as noted by Ch.K. Kang *et al.* (2019), in the future, the demand for an effective and safe anthrax vaccine will grow.

Thus, according to recent scientific studies, the use of extracellular toxins *B. anthracis* when creating new drugs is promising, and there is a need for further scientific developments on the prevention and treatment of anthrax.

Materials and Methods

The research was conducted in 2021 based on the Veterinary Laboratory of the State Scientific Institution “Centre for Innovative Biotechnologies” (Kyiv) under the programme No. 0119U100900 “Exotoxin of the causative agent of anthrax. Characteristics, identity between strains, alternative to antibiotic therapy”.

The following nutrient media were used in the study: nutrient broth (HIMEDIA) and nutrient agar (HIMEDIA), nutrient broth (GRM) –

Kesler medium based on fish meal hydrolysate, meat-peptone Agar (MPA), and meat-peptone broth (MPB). Vaccine strains of anthrax and anthrax microorganisms: *B. anthracis* K-79 Z, *B. anthracis* IBM-92Z (Institute of Veterinary Medicine, Kyiv), *B. anthracis* Sterne 34F 2, *B. anthracis* 55, *B. anthracis* SB, *B. anthracis* Tsenkovsky II, *B. cereus* 8035, *B. anthracoides* 67, *B. subtilis* BKM 17 (museum collection of microorganisms).

Microbiological, biotechnological, and statistical research methods were used in the process. The selection of strains was applied depending on the intensity of growth on nutrient media. A biotechnological method of cultivation and filtration was used to obtain a specific anthrax protein and examine the production of exotoxin by vaccine strains of the anthrax pathogen. The effect of pH on exotoxin production by experimental strains was determined by changes in the pH of the medium and the ability of different cultures to exhibit toxigenic properties. The HANNA H198128 pH meter was used. Experiments began with the production of pure working cultures of strains (Fig. 1-9), which were introduced to the HIMEDIA Agar (India) on Petri dishes and incubated at a temperature of 37°C for 48 hours.

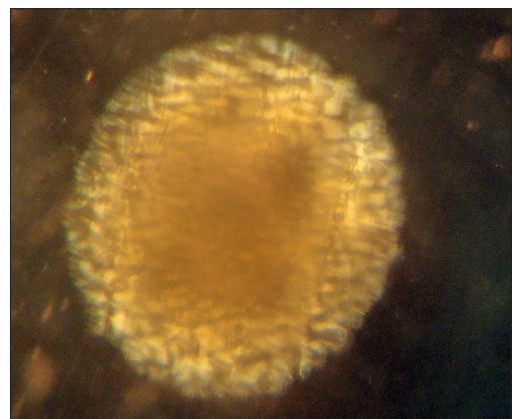


Figure 1. *B. anthracis* K-79 Z culture growth on nutrient agar (HIMEDIA)

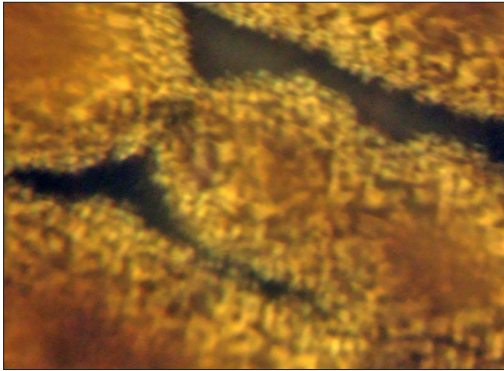


Figure 2. *B. anthracis* Tsenkovsky II (IBM92 Z) culture growth on nutrient agar (HIMEDIA)

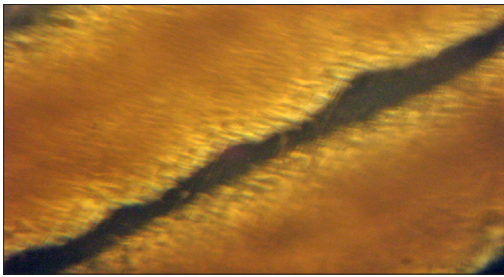


Figure 3. *B. anthracis* Sterne 34f2 culture growth on nutrient agar (HIMEDIA)

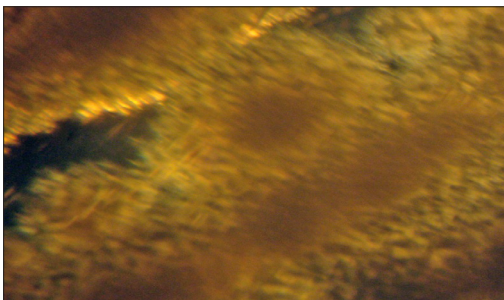


Figure 4. *B. anthracis* 55 culture growth on nutrient agar (HIMEDIA)

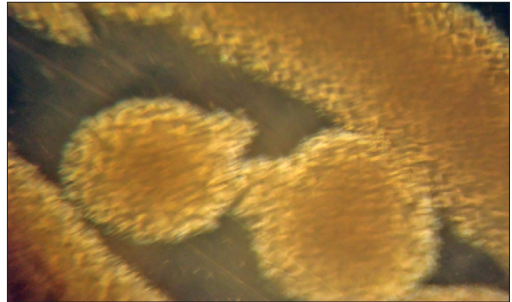


Figure 5. *B. anthracis* culture growth on nutrient agar (HIMEDIA)

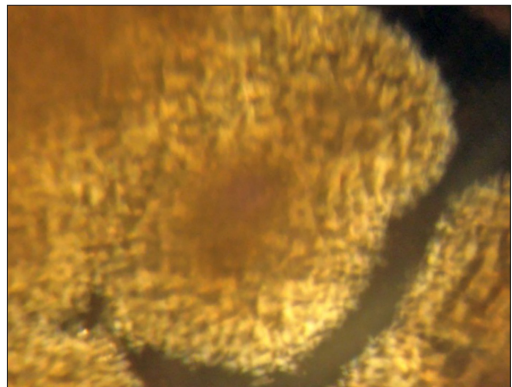


Figure 6. *B. anthracis* Tsenkovsky I culture growth on nutrient agar (HIMEDIA)

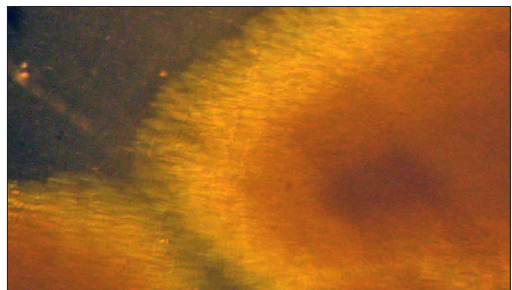


Figure 7. *B. cereus* 8035 culture growth on nutrient agar (HIMEDIA)

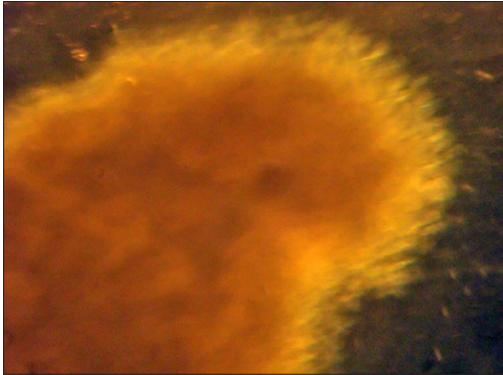


Figure 8. *B. anthracoides* 67 culture growth on nutrient agar (HIMEDIA)

After the first 24 hours of incubation, the cultures were checked under a microscope for the presence of alien microflora. After 48 hours, at the end of incubation on a solid medium, microscopy of the colonies was performed. The number of microbial cells was determined using the generally accepted method of counting the colony forming units (CFU) in veterinary medicine. At the end of incubation, it was washed off with a spatula on the nutrient broth (HIMEDIA).

Incubation was conducted for 48 hours in nutrient broth (HIMEDIA) at a temperature of $37.0 \pm 1^\circ\text{C}$. In the first 24 hours, the purity of crop growth was monitored. Grown colonies were selected using a bacteriological loop, and a “crushed drop” smear was applied and viewed under a microscope. pH control was conducted using a HANNA H198128 pH meter (Germany), the pH of the medium was 7.4. After testing, incubation was continued for up to 48 hours. At the end of incubation, exotoxins were obtained. Further, filtration through a bacteriological filter of the F5 brand (China) was conducted according to the author’s certificate No. 1022362.

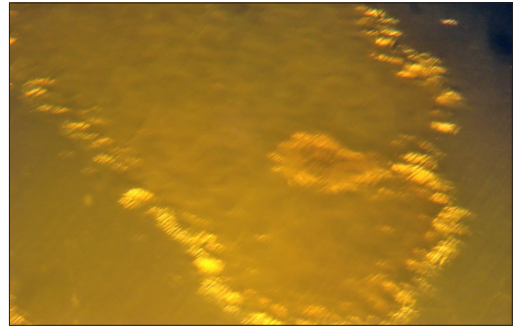


Figure 9. *B. subtilis* BKM 17 culture growth on nutrient agar (HIMEDIA)

Source: compiled by the author

The resulting filtrates were checked for contamination according to DSTU 4483:2005 (2006). The titer of anthrax protein was determined in the obtained exotoxins.

The precipitation disk reaction (RDP) was used to determine the titer of exotoxins. The disk-precipitation reaction was staged. The formulation of the disk-precipitation reaction is aimed at detecting in the culture liquid a specific for *B. anthracis* metabolite – exotoxin by immunodiffusion reaction in Agar gel using a specific anthrax serum. According to the Ascoli reaction formulation method, 0.5-1.0 cm³ opaque precipitation of anthrax serum was added to the bottom of a clean bacteriological test tube. A 1% agar gel, melted and cooled to 41-45°C, was layered on the serum using a Pasteur pipette along the wall of the test tube. After layering the Agar with a column 7-10 mm high, the test tube was left in an upright position for 15-20 minutes until it completely cooled down. The number of test tubes with such a system should correspond to the number of test tubes with sediment and

sediment dilutions. 1.0-2.0 cm³ of appropriate dilution of sediment and sediment was layered on the agar surface. The prepared test tubes were placed in a thermostat at a temperature of 37°C for 16-24 hours. The test tubes were visible in the penetrating light against a black background. The presence of a white precipitation line (disk) in the agar column was determined. The presence of a disk in the test tube indicated the presence of spenicific anthrax

protein. Precipitating anthrax serum (Kherson State Enterprise – Biological Dactory) was used in the reaction.

Results and Discussion

During experimental studies, the influence of changes in cultivation conditions on experimental strains of microbes of the *Bacillus* genus was determined. The results of the determination of exotoxin titers are presented in Table 1.

Table 1. Results of determination of anthrax protein titers in filtrates of anthrax pathogen strains during cultivation in nutrient broth (HIMEDIA)

Strain name	Colony-forming units (CFU)	Nutrient medium	Incubation temperature, °C	Incubation period, h	Titer of the resulting filtrate (genus)
<i>B. anthracis</i> K-79 Z	30×10 ⁶	HIMEDIA broth	37.0±1.0	48	1:64
<i>B. anthracis</i> IBM-92Z	31×10 ⁶				1:16
<i>B. anthracis</i> Sterne 34F ₂	32×10 ⁶				1:32
<i>B. anthracis</i> 55	30×10 ⁶				1:32
<i>B. anthracis</i> SB	29×10 ⁶				1:32
<i>B. anthracis</i> Tsenkovsky II.	31×10 ⁶				1:16
<i>B. cereus</i> 8035	30.2×10 ⁶				-
<i>B. anthracoides</i> 67	31×10 ⁶				-
<i>B. subtilis</i> BKM 17	29×10 ⁶				-

Source: compiled by the authors

Based on the results (Table 1), it can be seen that the strain *B. anthracis* K-79 Z, under identical growing conditions in a nutritious broth (HIMEDIA), produces an exotoxin with a titer of 1:64. Strains *B. anthracis* Sterne 34F₂, *B. anthracis* 55, and *B. anthracis* SB produced an exotoxin with a titer of 1:32. Therewith, it was established that the strains *B. anthracis* IBM-92Z and *B. anthracis* Tsenkovsky II produced an exotoxin with a specific anthrax protein titer of

1:16. Anthrax strains *B. cereus* 8035, *B. anthracoides* 67, and *B. subtilis* BKM 17 produced no anthrax exotoxin. The reaction for the genus was negative. Cultivation of virulent and avirulent strains of anthrax pathogen on nutritious GRM broth showed that the cultivation of vaccine strains of anthrax pathogen *B. anthracis* Sterne 34F₂, *B. anthracis* 55, and *B. anthracis* SB is less productive than the strain *B. anthracis* K-79 Z under the same conditions (Table 2).

Table 2. Results of determination of anthrax protein titers in filtrates of anthrax pathogen strains during cultivation on nutritious GRM broth

Strain name	Colony-forming units (CFU)	Nutrient medium	Incubation temperature, °C	Incubation period, h	Titer of the resulting filtrate
<i>B. anthracis</i> K-79 Z	28×10 ⁶	GRM broth	37.0±1.0	48	1:64
<i>B. anthracis</i> IBM-92Z	29×10 ⁶				1:16
<i>B. anthracis</i> Sterne 34F ₂	27×10 ⁶				1:32

Table 2. Continued

Strain name	Colony-forming units (CFU)	Nutrient medium	Incubation temperature, °C	Incubation period, h	Titer of the resulting filtrate
<i>B. anthracis</i> 55	28×10 ⁶	GRM broth	37.0±1.0	48	1:32
<i>B. anthracis</i> SB	30×10 ⁶				1:32
<i>B. anthracis</i> Tsenkovsky II.	27×10 ⁶				1:16
<i>B. cereus</i> 8035	28×10 ⁶				-
<i>B. anthracoides</i> 67	31×10 ⁶				-
<i>B. subtilis</i> BKM 17	29×10 ⁶				-

Source: compiled by the authors

As can be seen from the results (Table 2), the lowest titer of exotoxin when incubated in GRM broth corresponded to *B. anthracis* IBM-92 Z and *B. anthracis* Tsenkovsky II, the titer of exo-

toxin in the RDP reaction was 1:16. The results of growing crops on HIMEDIA broth with the addition of 40% glucose solution in the amount of 5% are presented in Table 3.

Table 3. Results of determination of anthrax protein titers in filtrates of anthrax pathogen strains when cultured in nutrient broth (HIMEDIA) + glucose 5%

Strain name	Colony-forming units (CFU)	Nutrient medium	Incubation temperature, °C	Incubation period, h	Filtrate titer
<i>B. anthracis</i> K-79 Z	31×10 ⁶	HIMEDIA broth+glucose 5%	37.0±1.0	48	1:128
<i>Bac. anthracis</i> IBM-92Z	29×10 ⁶				1:16
<i>B. anthracis</i> Sterne 34F ₂	30×10 ⁶				1:32
<i>B. anthracis</i> 55	30×10 ⁶				1:32
<i>B. anthracis</i> SB	30×10 ⁶				1:32
<i>B. anthracis</i> Tsenkovsky II.	29×10 ⁶				1:16
<i>B. cereus</i> 8035	28×10 ⁶				-
<i>B. anthracoides</i> 67	31×10 ⁶				-
<i>B. subtilis</i> BKM 17	31×10 ⁶				-

Source: compiled by the authors

The research shown in Table 3 indicate that the titer of the exotoxin strain *B. anthracis* K-79 Z when grown in HIMEDIA + 5% glucose broth was 1:128. This indicator is the highest among vaccine strains of the anthrax pathogen. Changes in cultivation conditions did not affect the production of exotoxin by vaccine strains *B. anthracis* Sterne 34F₂, *B. anthracis* 55, *B. anthracis* SB, and the exotoxin titer was 1:32. Exotoxins of strains *B. anthracis* IBM-92Z and *B. anthracis* Tsenkovsky II had a specific protein titer of 1:16. Saprophytic spore-forming bacilli *B. anthracoides* 67, *B. subtilis* 17, and *B. cereus* 8035

do not produce a specific toxin, and therefore, their filtrates of culture fluids did not react by precipitation (Ascoli) with antibodies of precipitating anthrax serum.

The examination of the effect of the pH of the medium on the production of exotoxin of anthrax strains began with the production of a bacterial mass with an identical number of microbial cells – 30.2 - 30.8 × 10⁶ CFU. Strains were introduced in the amount of: *B. anthracis* K-79 Z - 30.5 × 10⁶, *B. anthracis* IBM-92Z – 30.7 × 10⁶, *B. anthracis* Sterne 34F₂ – 30.2 × 10⁶, *B. anthracis* 55 – 30.8 × 10⁶, *B. anthracis* SB – 30.5 × 10⁶

and *B. anthracis* Tsenkovsky II – 30.2×10^6 . Colonies grown on MPA were introduced in a liquid nutrient medium – MPB with different pH values – 6.5; 8.5; 7.5. Incubation was conducted in a thermostat for 48 hours at a temperature of 37°C. The bacterial mass was filtered through F5 grade bacterial filters. The obtained filtrates were checked according to DSTU 4483:2005 (2006). With filtrates of the culture liquid, RDP was applied to determine the titer of the anthrax protein. According to the results of the study, it was determined that vaccine strains produce different amounts of exotoxin when the pH of the medium changes.

According to the results of the experiment, it was found that *B. anthracis* K-79 Z when cultured at pH 6.5, showed an exotoxin titer according to RDP – 8, when cultured at pH 7.5, the exotoxin titer was 64, and when cultured at pH 8.5-32. The average titer of the resulting exotoxin corresponded to a value of $1:34.66 \pm 16.22$. Culture *B. anthracis* IBM-92 Z, when cultured at pH 6.5, showed an exotoxin titer of 4, and when incubated at pH 7.5-8.0. If the pH of the medium changed to 8.5, the exotoxin titer corresponded to 4. The average titer of exotoxin strain *B. anthracis* IBM-92 Z was equal to $1:5.33 \pm 1.33$. When the pH value of the medium changes to 6.5, the vaccine strain *B. anthracis* Sterne 34F2 showed the title 8. When cultured on a medium at pH 7.5, the exotoxin titer corresponded to 32. When cultured at pH 8.5, a titer of 1:16 was obtained. The average exotoxin score was $1: 18.66 \pm 7.05$.

Exotoxin production indicators also changed in *B. anthracis* 55. When cultured at pH 6.5, exotoxin production was 1:8 in titer. If the pH value of the medium changes to 7.5, an exotoxin titer of 1:32 is obtained. For changes in the pH of the medium to 8.5, an exotoxin titer of 1:16 was noted. Average titer of exotoxin *B. anthracis* 55 in the experiment was $1:18.66 \pm 7.05$. Vaccine strain *B. anthracis* SB, when cultured on a nutrient medium, a pH value of 6.5 showed a

titer of anthrax protein of 1:8. When cultured at a medium pH of 7.5, an exotoxin with a titer of 1:32 was obtained. When the pH of the medium changed to 8.5, an exotoxin was noted in the titer of 1:16. The average exotoxin production of the strain *B. anthracis* SB was $1: 18.66 \pm 7.05$.

Culture *B. anthracis* Tsenkovsky II, when cultured at pH 6.5, showed an exotoxin titer of 1:8. When cultured at pH 7.5, the exotoxin titer was 1:16. When cultured at a medium pH of 8.5, an exotoxin titer of 1:8 was obtained. Average exotoxin production of the strain *B. anthracis* Tsenkovsky II corresponded to $1:10.66 \pm 2.66$. The results of experimental studies show that during the cultivation of vaccine strains of anthrax at different pH values of the medium, the content of a specific protein in exotoxins varied. Despite changes in the pH value of the medium, the highest titer of exotoxin was obtained from the strain *B. anthracis* K-79Z, the average amount of specific anthrax protein in the bacterial mass filtrate was $1:34.66 \pm 16.22$. Notably, at pH values of 8.5 of the medium, *B. anthracis* K-79Z produced exotoxin in a titer of 1:32. This is the highest rate of formation of a specific anthrax protein among the experimental strains at a pH of 8.5 of the medium.

According to the results, it was established that the production of exotoxin can change with fluctuations in the pH values of the medium, which has practical application in the development of a biotechnological process for manufacturing a vaccine from a strain or creating diagnosticums as, for example, a standard anthrax antigen. Changing the pH value of the medium allows correcting the biotechnological process of spore formation, the intensity of spore germination, the formation and accumulation of exotoxin in the cultural fluid. In accordance with the literature data, the research on the exotoxin production of anthrax pathogen strains is relevant. Typically, studies use one or two strains to produce vaccines or

diagnosticums. This study compares the vaccine strains used for the production of vaccines for veterinary medicine in Ukraine and abroad. Therewith, over the past five years, comparative studies of such vaccine strains regarding the production of exotoxin and the influence of external factors on toxin formation have not been conducted by other researchers.

M.H. Norris *et al.* (2020) analysed the spore formation of field and vaccine strains of the anthrax pathogen in the laboratory using nutrient media. The number of viable spores was taken into account according to the CFU. The authors confirmed that laboratory strains grown on media without animal protein quickly lose their ability to spore. Laboratory strains *B. anthracis* produce fewer proteins associated with growth and spore formation compared to wild strains isolated as a result of zoonotic diseases. According to the results of the paper, the rate of their growth on nutrient media is more slowed down than in field strains. It was also confirmed that wild strains grow faster than laboratory strains, showing greater sensitivity to the availability of nutrients. Spore formation in wild strains was substantially faster compared to laboratory strains, which indicates the ability of wild strains to accumulate spores faster due to nutrient restrictions. Meanwhile, in laboratory strains, there is a decrease in the rate at which they use nutrients and an increase in the time of spore formation. In addition, the authors noted that the mechanisms of growth retardation in laboratory strains require further investigation. Therefore, studies on the influence of the medium and cultivation conditions of vaccine strains are relevant and have a practical need.

D. Galante *et al.* (2022) noted that PA plays a crucial role in the immune inactivation of anthrax toxins, as it induces the production of neutralising antibodies that are necessary to provide protection against anthrax. The

researchers proved a clear correlation between neutralising antibodies and protective immunity and highlighted the dependence of the effectiveness of anthrax vaccines on the magnitude of the humoral anti-PA response induced in animals or humans. In addition to toxin proteins, in media containing bicarbonate and providing 5% CO₂, which happens when mammalian tissues are infected, *B. anthracis* secretes many other proteins that play an important role in the virulence process. Therefore, proteases and degrading enzymes were identified that can inactivate proteins, cleave host antimicrobial factors, and modify secreted bacterial virulence factors, thereby performing additional functions for the complete virulence of *B. anthracis*.

As noted by A. Verma & D.L. Burns (2018) and Ch.K. Kang *et al.* (2019), the demand for an effective and safe anthrax vaccine is growing as humanity prepares for possible bioterrorism in the future. However, the currently adsorbed anthrax vaccine (AVA, BioThrax TM, Lansing, Michigan) remains the only U.S. FDA-approved vaccine that has been researched for decades. The authors noted that the optimal scheme and route of administration of AVA is still unknown and expressed several reservations about its pharmaceutical quality and consistency from batch to batch, which requires the creation of a new anthrax vaccine. Therewith, a randomised, blind, placebo-controlled phase II clinical trial was conducted on healthy adult volunteers to assess the immunogenicity and safety of a new anthrax vaccine with recombinant protective antigen (rPA), GC1109. The new anthrax vaccine rPA GC1109 was identified to have immunogenic activity after three doses of intramuscular administration and was well tolerated.

J.B. Felix *et al.* (2020) proved that the Sterne vaccine cannot elicit an immune response after oral vaccination. This vaccine contains a suspension of live attenuated spores of *B. anthracis* strain Sterne 34f2 (Sterne spores) in saponin

and is a modern injectable vaccine for livestock. When administered orally, it is ineffective, and individual subcutaneous injections are not a practical method of vaccination of wild animals. Based on the conducted studies, the authors established a direct dependence of the effectiveness of the drug on the pH of the medium and the effect on the vaccine strain when creating various cultivation conditions, which confirms the results described in this paper. The stability of Sterne spores at different pH values was evaluated *in vitro*, and it was determined that the medium at pH 2 leads to the death of spores of the vaccine strain. The authors confirmed that protection from the stomach environment is a major problem in the production of oral vaccines. Therefore, there is a need to create an effective oral anthrax vaccine for wild animals. An oral anthrax vaccine is urgently needed to prevent annual anthrax outbreaks that lead to catastrophic losses of livestock and wildlife around the world.

V. Savransky *et al.* (2019) noted that existing licensed preventive vaccines are heterogeneous products and may include inactivated bacterial and viral vaccines, live attenuated vaccines, recombinant proteins, polysaccharide and conjugated vaccines, and DNA vaccines. They often contain immunostimulating components represented by various adjuvants and require developing new anthrax vaccines designed to improve the currently licensed one. These improvements include fewer vaccinations and faster protection. V. Savransky *et al.* (2019) indicated that rPA7909 is an anthrax vaccine with recombinant protective antigen (rPA) supplemented with the immunostimulating oligonucleotide CpG 7909 and was developed for post-exposure anthrax prevention. The vaccine is a sterile lyophilised form, which, after dilution, has the appearance of a suspension from white to almost white in color. The final reconstituted drug contains 75 mcg of rPA, 750 mcg of aluminium (as an adjuvant of

algidrogel), and 250 mcg of CpG 7909 in 0.5 mL (full dose) with other excipients. The rPA7909 vaccine was well tolerated by the animal body and caused local and systemic effects associated with the inflammatory process provoked by the injected material. The expected antigen-specific antibody response was observed in animals that received the rPA7909 vaccine.

M.A. Smiley *et al.* (2019), D. Żakowska *et al.* (2019), and D.R. Weilhammer *et al.* (2020) presented a study on mutant variants of the PA exotoxin used to improve vaccines. T.B. Gallagher *et al.* (2019) proved that PA is the main protective immunogen in anthrax vaccines licensed in the UK and US. The use of recombinant protective antigen (rPA) (rPA7909) as a post-exposure anthrax prevention was proven by V. Savransky *et al.* (2019). Anthrax vaccine production in the UK (AVP) is focused on reproducing protective antigen (PA) from the *Bacillus anthracis* Sterne strain. However, some fundamental properties of AVP still need to be examined. Therefore, it is necessary to investigate the differences in degrees of protection in different animal species, even though AVP has been used for decades, as noted by T. Modi *et al.* (2021).

S. Jauro *et al.* (2020) noted that the vaccine from the *Bacillus anthracis* Sterne strain with live spores (SLSV, strain *Bacillus anthracis* 34F2) is an effective veterinary anthrax vaccine, although its use with antimicrobials is contraindicated. However, the use of a non-live anthrax vaccine (NLAV) may overcome the limitations of SLSV. The authors presented the results of a study on cattle vaccinated with either NLAV (purified recombinant PA (PrPA) or rPA (CrPA) and formaldehyde-inactivated spores (strain FIS *B. anthracis* 34f2) and emulsigen-D®/Alhydrogel® adjuvant. The potential of NLAV (PrPA+FIS or CrPA+FIS) applied together with combined adjuvants (Emulsigen-D®/Alhydrogel®) to induce an immune response against toxins and spores *B. anthracis* in vaccinated cattle

was established. Immune response with Pr-PA+FIS+Emulsigen-D®/Alhydrogel® demonstrated a substantial level of protection in the passive protection analysis on mice, which is comparable to SLSV. Due to the inanimate property of the vaccine, it is compatible with antibiotic treatment in the event of an outbreak. It can be used for vaccination of wild animals and on grazing grounds. It lacks residual virulence in vaccinated animals. Field clinical trials of the vaccine are currently underway. The use of antigens as components of new drugs against anthrax is a promising direction in improving methods of control and Prevention of this disease in animals.

H. Mirhaj *et al.* (2019) noted that the standard approach to anthrax therapy is to destroy germinating bacilli by injecting aggressive antibiotics. However, antibiotic therapy is ineffective when systemic symptoms of anthrax appear since, by this time, deadly concentrations of anthrax toxin accumulate in the body of sensitive animal species, and as a result of natural evolution, antibiotic-resistant strains appear. Deliberate modification through genetic engineering is also a new challenge for traditional antibiotic treatment. Therefore, the development of an antitoxin for combined use with antibiotic therapy is a top priority. Modern vaccines are produced in England and the United States on the basis of precipitation of cell extracts. The production of next-generation anthrax vaccines focuses on various recombinant expression systems. As a result of this study, it was determined that antibodies generated against four PA regions (PAD4) can neutralise anthrax toxin and PA mixed with LF, which increases the specific antibody response to PA. Thus, from the available literature sources, it is known about the research on toxin formation of a vaccine strain *B. anthracis Sterne 34F₂* used for the production of anthrax vaccines in the United States and England.

In Ukraine, biofactories produce veterinary preventive drugs from three strains: *B. anthracis Sterne 34F₂*, *B. anthracis K-79Z*, and *B. anthracis SB*. The creation of vaccines is based on the spore mass of the anthrax pathogen. Foreign developments are based on the products of *B. anthracis* strain metabolism and are mostly aimed at preventing infection in humans. Academician A.I. Zaviryukha and a team of scientists from the relevant laboratory of the Institute of Molecular Biology and Genetics of the National Academy of Sciences of Ukraine (Kyiv) commenced the examination and practical application of exotoxins of infectious diseases pathogens in animals in the composition of biological preparations. The selection and deposition of strains producing toxins was conducted: anthrax (strains *B. anthracis K-79 Z* and *IBM 92 Z*), colibacteriosis (*E. coli IBM-1*), pasteurellosis (*P. multocida 84z*), salmonellosis. The possibility of using anthrax exotoxins in the manufacture of specific diagnosticums and inactivated immunogenic vaccines were experimentally proven. The results presented in this paper are a continuation of research on the development of modern technologies for obtaining exotoxins of pathogenic microorganisms and their determination, presence, and quantity in the culture fluid and elucidation of their effect on the immune system of animals.

The authors of this paper consider all the above aspects regarding preventive drugs from the vaccine strain *B. anthracis Sterne 34F₂*, especially its properties related to the formation of exotoxin. Due to the museum of microorganisms, in particular, the presence of the aforementioned strains of anthrax and anthrax bacilli, researchers have the opportunity to compare the parameters of cultures of the *Bacillus* genus. Long-term work with various reference strains indicates substantial advantages of the vaccine strain *B. anthracis K-79 Z* regarding the production of exotoxin in comparison

with *B. anthracis* Sterne 34F₂, *B. anthracis* 55, and *B. anthracis* SB, which is the main source of immunogenicity of the vaccine, namely the effectiveness of its use for animals and humans. At the present stage of creating vaccines for effective anthrax prevention, it is vital to use anthrax pathogens with a lower allergenic effect and the ability to preserve the stability of exotoxin as a source of immunogenicity. Therefore, examining vaccine strains at different stages of the creation of anti-selective drugs is a relevant direction of scientific and practical search.

Conclusions

On the basis of the Veterinary Laboratory of the State Scientific Institution “Centre for Innovative Biotechnologies”, a study of the ability of various microbes of the *Bacillus* genus to produce exotoxins outside the cell wall when culture conditions change during incubation was conducted. It was established that when the cultivation conditions and pH values of the medium change, vaccine strains of the anthrax pathogen produce different amounts of exotoxin. The *B. anthracis* K-79 Z vaccine strain was the most resistant to changes in the pH of the medium, the average amount of specific anthrax protein in the bacterial mass filtrate was 1: 34.66±16.22. Cultivation in a medium with a pH value of 7.5 was the most optimal for strains *B. anthracis* Sterne 34F₂, *B. anthracis* 55, and *B. anthracis* SB. The titer of the resulting anthrax protein was 1:32. Under the same cultivation conditions with the experimental vaccine strains and a certain favourable pH 7.5 of the medium, the highest titer of a specific anthrax protein was 1:64, obtained from the bacilli of the vaccine strain *B. anthracis* K-79 Z. It was determined that a change in the pH of the medium to 8.5 provoked experimental strains of the anthrax pathogen to produce exotoxin in the titer of a specific anthrax protein – 1:16, which is much lower than that under optimal

cultivation conditions. Change in the incubation pH values of the nutrient medium to 6.5 affected the growth properties of strains and the process of toxin formation, which manifested itself in a decrease in the production of specific anthrax protein to 1:8 in filtrates of bacterial masses of vaccine strains *B. anthracis* Sterne 34F₂, *B. anthracis* 55, and *B. anthracis* SB. When the cultivation conditions changed – an increase in the pH of the medium to 8.5, the experimental strains showed a similar decrease in the production of a specific anthrax protein, which was 1:32. This indicates similar characteristics of these vaccine strains and an identical effect of the pH value of the medium on exotoxin production. Under the same cultivation conditions with the experimental vaccine strains and a certain favourable pH 7.5 of the medium, the highest titer of a specific anthrax protein was 1:64, obtained from the bacilli of the vaccine strain *B. anthracis* K-79 Z.

The presence of high rates of exotoxin production in vaccine strains of anthrax pathogen is an important indicator in the development of new drugs against anthrax pathogen for use in veterinary medicine. In the future, studies of toxigenic properties of vaccine strains of the anthrax pathogen will be conducted and their further use in veterinary medicine during the development of new preventive drugs for anthrax, which will contribute to the epizootic security of the state.

Acknowledgements

The team of authors expresses their gratitude to V.M. Bobyliov, a researcher at the Institute of Molecular Biology and Genetics of the National Academy of Sciences of Ukraine, who conducted experiments on the selection of microorganisms.

Conflict of Interest

None.

References

- [1] Alameh, S., Bartolo, G., O'Brien, S., Henderson, E.A., Gonzalez, L.O., Hartmann, S., Klimko, C.P., Shoe, J.L., Cote, C.K., Grill, L.K., Levitin, A., & Martchenko, Sh.M. (2020). Anthrax toxin component, Protective Antigen, protects insects from bacterial infections. *PLoS ONE*, 16(8), article number 1008836. doi: [10.1371/journal.ppat.1008836](https://doi.org/10.1371/journal.ppat.1008836).
- [2] Asheneffe, W., Fantaw, S., Mekonene, Y., Teshale, A., Yitagesu, Y., Tsige, E., Getahun, D., Geremew, T., Abichu, G., Moges, B., Abate, E., Abayneh, T., Zeru, T., Belay, Z., & Mor, S. (2022). First PCR Confirmed anthrax outbreaks in Ethiopia-Amhara region, 2018-2019. *PLOS Neglected Tropical Diseases*, 16(2), article number e0010181. doi: [10.1371/journal.pntd.0010181](https://doi.org/10.1371/journal.pntd.0010181).
- [3] Crawford, T., Fletcher, N., Veitch, M., Gonzalez, J.L., Pett, N., Brereton, I., Wells, J.W., Mobli, M., & Tesiram, Y. (2019). *Bacillus anthracis* protective antigen shows high specificity for a UV induced mouse model of cutaneous squamous cell carcinoma frontiers in medicine (Lausanne). *Frontiers in Medicine*, 6, article number 22. doi: [10.3389/fmed.2019.00022](https://doi.org/10.3389/fmed.2019.00022).
- [4] Dale, J.L., Raynor, M.J., Ty, M.C., Hadjifrangiskou, M., & Koehler, T.M. (2018). A dual role for the bacillus anthracis master virulence regulator AtxA: Control of sporulation and anthrax toxin production. *Frontiers in Microbiology*, 9, article number 482. doi: [10.3389/fmicb.2018.00482](https://doi.org/10.3389/fmicb.2018.00482).
- [5] Diaz-Arévalo, D., Chen, Y., & Zeng, M. (2021). Vaccine delivery with a detoxified bacterial toxin, methods in molecular biology, 2183, 423-435. doi: [10.1007/978-1-0716-0795-4_22](https://doi.org/10.1007/978-1-0716-0795-4_22).
- [6] DSTU 4483:2005. (2006). *Veterinary immunobiological preparations. Methods of determining bacterial and fungal contamination*. Retrieved from <http://surl.li/qqvrj>.
- [7] Dumas, E.K., Demiraslan, H., & Ingram, R.J., Sparks, R.M., Muns, E., Zamora, A., Larabee, J., Garman, L., Ballard, J.D., Boons, G.J., James, J.A., Kayabas, U., Doganay, M., & Darise Faris, A. (2020). Toxin neutralizing antibodies elicited by naturally acquired cutaneous anthrax are elevated following severe disease and appear to target conformational epitopes. *PLoS ONE*, 15(4), article number e0230782. doi: [10.1371/journal.pone.0230782](https://doi.org/10.1371/journal.pone.0230782).
- [8] Felix, J.B., Chaki, S.P., Xu, Y., Ficht, T.A., Rice-Ficht, A.C., & Cook, W.E. (2020). Protective antibody response following oral vaccination with microencapsulated *Bacillus Anthracis* Sterne strain 34F2 spores. *NPJ Vaccines*, 5, article number 59. doi: [10.1038/s41541-020-0208-3](https://doi.org/10.1038/s41541-020-0208-3).
- [9] Fischer, E.S., Campbell, W.A., Liu, Sh., Ghirlando, R., Fattah, R.J., Bugge, T.H., & Leppla, S.H. (2019). Bismaleimide cross-linked anthrax toxin forms functional octamers with high specificity in tumor targeting. *Protein Science*, 28(6), 1059-1070. doi: [10.1002/pro.3613](https://doi.org/10.1002/pro.3613).
- [10] Frydrych, J., Skácel, J., Šmídková, M., Mertlíková-Kaiserová, H., Dračínský, M., Gnanasekaran R., Lepšík, M., Soto-Velasquez, M., Watts, V.J., & Janeba, Z. (2018). Synthesis of α -branched acyclic nucleoside phosphonates as potential inhibitors of bacterial adenylate cyclases. *ChemMedChem*, 13(2), 199-206. doi: [10.1002/cmdc.201700715](https://doi.org/10.1002/cmdc.201700715).
- [11] Fonseca, J.M., Mackowiak da Fonseca, I.I., Nagamine, M.K., Oliveira Massoco, C., Nishiya, A.T., Ward, J.M., Liu, Sh., Leppla, S.H., Bugge, T.H., & Dagli, M.L. (2020). Inhibitory effects of a reengineered anthrax toxin on canine and human osteosarcoma cells. *Toxins (Basel)*, 12(10), article number 614. doi: [10.3390/toxins12100614](https://doi.org/10.3390/toxins12100614).
- [12] Gallagher, T.B., Mellado-Sanchez, G., Jorgensen, A.L., Moore, S., Nataro, J.P., Pasetti, M.F., & Baillie, L.W. (2019). Development of a multiple-antigen protein fusion vaccine candidate that confers protection against *Bacillus anthracis* and *Yersinia pestis*. *PLOS Neglected Tropical Diseases*, 13(8), article number e0007644. doi: [10.1371/journal.pntd.0007644](https://doi.org/10.1371/journal.pntd.0007644).

- [13] Galante, D., Manzulli, V., Donatiello, A., Fasanella, A., Chirullo, B., Francia, M., Rondinone, V., Serrecchia, L., Pace, L., Iatarola, M., Tarantino, M., & Adone, R. (2022). Production of a *Bacillus anthracis* secretome with suitable characteristics as antigen in a complement fixation test. *Life*, 12(2), article number 312. doi: [10.3390/life12020312](https://doi.org/10.3390/life12020312).
- [14] Jauro, S., Ndumnego, O.C., Ellis, Ch., Buys, A., Beyer, W., & Heerden, H. (2020). Immunogenicity of non-living anthrax vaccine candidates in cattle and protective efficacy of immune sera in A/j mouse model compared to the Sterne live spore vaccine. *Pathogens*, 9(7), article number 557. doi: [10.3390/pathogens9070557](https://doi.org/10.3390/pathogens9070557).
- [15] Kang, Ch.K., Kim, N.H., Kim, Ch.J., Rhie, Gi.E., Jo, S.K., Ahn, M., Kang, J., Choe, P.G., Park, W.B., Kim, N.J., & Oh, M.D. (2019). Immunogenicity and safety of a novel recombinant protective antigen anthrax vaccine (GC1109), a randomized, single-blind, placebo controlled phase II clinical study. *Vaccine*, 37(29), 3820-3824. doi: [10.1016/j.vaccine.2019.05.057](https://doi.org/10.1016/j.vaccine.2019.05.057).
- [16] Kisaakye, E., Rioplexus Ario, A., Bainomugisha, K., Cossaboom, C., Lowe, D., Bulage, L., Kadobera, D., Sekamatte, M., Lubwama, B., Tumusiime, D., Tusiime, P., Downing, R., Buule, J., Lutwama, J., Salzer, J.S., Matkovic, E., Ritter, J., Gary, J., & Zhu, B. (2020). Outbreak of anthrax associated with handling and eating meat from a cow, Uganda, 2018. *Emerging Infectious Diseases*, 26(12), 2799-2806. doi: [10.3201/eid2612.191373](https://doi.org/10.3201/eid2612.191373).
- [17] Kondakova, O.A., Nikitin, N.A., Evtushenko, E., Ryabchevskaya, E.M., Atabekov, J.G., & Karpova, O.V. (2019). Vaccines against anthrax based on recombinant protective antigen: Problems and solutions. *Expert Review of Vaccines*, 18(8), 813-828. doi: [10.1080/14760584.2019.1643242](https://doi.org/10.1080/14760584.2019.1643242).
- [18] Liddington, R.C. (2021). Assembly and Function of the anthrax toxin protein translocation complex. *Subcellular Biochemistry*, 96, 563-577. doi: [10.1007/978-3-030-58971-4_18](https://doi.org/10.1007/978-3-030-58971-4_18).
- [19] Liu, W., & Nestorovich, E.M. (2021). Anthrax toxin channel: What we know based on over 30 years of research. *Biochimica et Biophysica Acta*, 1863(11), article number 183715. doi: [10.1016/j.bbamem.2021.183715](https://doi.org/10.1016/j.bbamem.2021.183715).
- [20] Manish, M., Verma, Sh., Kandari, D., Kulshreshtha, P., Singh, S., & Bhatnagar, R. (2020). Anthrax prevention through vaccine and post-exposure therapy. *Expert Opinion on Biological Therapy*, 20(12), 1405-1425. doi: [10.1080/14712598.2020.1801626](https://doi.org/10.1080/14712598.2020.1801626).
- [21] Mirhaj, H., Honari, H., & Zamani, E. (2019). [Evaluation of immune response to recombinant Bacillus anthracis LFD1-PA4 chimeric protein](#). *Iranian Journal of Veterinary*, 20(2), 112-119.
- [22] Modi, T., Gervais, D., Smith, S., Miller, J., Subramaniam, Sh., Thalassinis, K., & Shepherd, A. (2021). Characterization of the UK anthrax vaccine and human immunogenicity. *Human Vaccines & Immunotherapeutics*, 17(3), 747-758. doi: [10.1080/21645515.2020.1799668](https://doi.org/10.1080/21645515.2020.1799668).
- [23] Norris, M.H., Zincke, D., Leiser, O.P., Kreuzer, H., Hadfield, T.L., & Blackburn, J.K. (2020). *Laboratory strains of Bacillus anthracis lose their ability to rapidly grow and sporulate compared to wildlife outbreak strains*. Retrieved from <https://pubmed.ncbi.nlm.nih.gov/31978128/>.
- [24] Omodo, M., Gardela, J., Namatovu, A., Okurut, A., Esau, M., Acham, M., Nakanjako, M., Israel, M., Isingoma, E., Moses, M., Paul, L., Ssenkeera, B., Atim, S., Gonahasa, D., Sekamatte, M., Gouilh, M., & Gonzalez, J. (2023). Anthrax bio-surveillance of livestock in Arua District, Uganda, 2017-2018. *Acta Tropica*, 240, article number 106841. doi: [10.1016/j.actatropica.2023.106841](https://doi.org/10.1016/j.actatropica.2023.106841).

- [25] Pilo, P., & Frey, J. (2018). Pathogenicity, population genetics and dissemination of *Bacillus anthracis*. *Infection, Genetics and Evolution*, 64, 115-125. doi: [10.1016/j.meegid.2018.06.024](https://doi.org/10.1016/j.meegid.2018.06.024).
- [26] Savransky, V., Lacy, M., Ionin, B., Skiadopoulos, M.H., & Shearer, J. (2019). Toxicity study of a lyophilized recombinant protective antigen-based anthrax vaccine adjuvanted. *International Journal of Toxicology*, 38(3), 163-172. doi: [10.1177/1091581819848722](https://doi.org/10.1177/1091581819848722).
- [27] Simonsen, K.A., & Chatterjee, K. (2019). *Anthrax*. Retrieved from <https://www.ncbi.nlm.nih.gov/books/NBK507773/>.
- [28] Smiley, M.A., Sanford, D.C., Triplett, Ch.A., Callahan, D., Frolov, V., Look, J., Ruiz, Ch., Reece, J.J., Miles, A., Ruiz, E., Ionin, B., Shearer, J.D., & Savransky, V. (2019). Comparative immunogenicity and efficacy of thermostable (lyophilized) and liquid formulation of anthrax vaccine candidate AV7909. *Vaccine*, 37(43), 6356-6361. doi: [10.1016/j.vaccine.2019.09.015](https://doi.org/10.1016/j.vaccine.2019.09.015).
- [29] Sumithra, T.G., Chaturvedi, V.K., Gupta, P.K., Bincy, J., Siju, S.J., Sunita, S.C., Reshma, K.J., Patel, C.L., & Rai, A.K. (2021). A novel bicistronic DNA vaccine with enhanced protective immune response against *Bacillus anthracis* through DNA prime-protein boost vaccination approach. *Microbial Pathogenesis*, 158, article number 105104. doi: [10.1016/j.micpath.2021.105104](https://doi.org/10.1016/j.micpath.2021.105104).
- [30] Topluoglu, S., Aktas, D., Celebi, B., Kara, F., Doganay, M., & Alp, E. (2021). Human anthrax in Turkey: A ten years' experience (2009-2018). *Tropical Doctor*, 51(1), 80-83. doi: [10.1177/0049475520969542](https://doi.org/10.1177/0049475520969542).
- [31] Tournier, J., & Rougeaux, C. (2020). Anthrax toxin detection: From *in vivo* studies to diagnostic applications. *Microorganisms*, 8(8), article number 1103. doi: [10.3390/microorganisms8081103](https://doi.org/10.3390/microorganisms8081103).
- [32] Verma, A., & Burns, D.L. (2018). *Improving the stability of recombinant anthrax protective antigen vaccine*. Retrieved from <https://pubmed.ncbi.nlm.nih.gov/30228030/>.
- [33] Walker, M.A., Uribasterra, M., Asher, V., Getz, W.M., Ryan, S.J., Ponciano, J.M., & Blackburn, J.K. (2021). Anthrax surveillance and the limited overlap between obligate scavengers and endemic anthrax zones in the United States. *Vector-Borne and Zoonotic Diseases*, 21(9), 675-684. doi: [10.1089/vbz.2020.2747](https://doi.org/10.1089/vbz.2020.2747).
- [34] Weilhammer, D.R., Dunkle, A.D., Boone, T., Gilmore, S.F., Khemmani, M., Peters, S.K.G., Hoepflich, P.D., Fischer, N.O., Blanchette, C.D., Driks, A., & Rasley, A. (2020). Characterization of *Bacillus anthracis* spore proteins using a nanoscaffold vaccine platform. *Frontiers in Immunology*, 11, article number 1264. doi: [10.3389/fimmu.2020.01264](https://doi.org/10.3389/fimmu.2020.01264).
- [35] Young, C.J., Richard, K., Beruar, A., Lo, S.Y., & Siemann, S. (2018). An investigation of the pH dependence of copper-substituted anthrax lethal factor and its mechanistic implications. *Journal of Inorganic Biochemistry*, 182, 1-8. doi: [10.1016/j.jinorgbio.2018.01.015](https://doi.org/10.1016/j.jinorgbio.2018.01.015).
- [36] Żakowska, D., Graniak, G., Rutyna, P., Naylor, K., Głowacka, P., & Niemcewicz, M. (2019). Protective antigen domain 4 of *Bacillus anthracis* as a candidate for use as vaccine for anthrax. *Annals of Agricultural and Environmental Medicine*, 26(3), 392-395. doi: [10.26444/aaem/99669](https://doi.org/10.26444/aaem/99669).

Особливості продукції екзотоксину вакцинних штамів збудника сибірки для застосування у ветеринарній галузі

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Анотація. Основним джерелом імуногенності протисибіркових вакцин, які застосовуються у ветеринарній медицині є екзотоксини, що продукують вакцинні штами збудника сибірки. Актуальність дослідження зумовлена пошуком найпридатніших до використання вакцинних штамів збудника сибірки, з метою отримання високої продукції екзотоксину, як фактора ефективності препаратів для здійснення профілактичних і безпекових заходів у галузі ветеринарії. У зв'язку з цим, мета роботи полягала у дослідженні продуктивних властивостей мікробів роду *Bacillus* щодо продукції екзотоксину за зміни умов культивування під час інкубації. При дослідженні вакцинних штамів збудника сибірки використовували мікробіологічні та біотехнологічні методи, а також порівняльний статистичний аналіз. Проводили відбір штамів за інтенсивністю росту на поживних середовищах. Для вивчення продукції екзотоксину вакцинними штамми збудника антракса застосовували біотехнологічний підхід отримання специфічного сибіркового білка. При культивуванні на ідентичних поживних середовищах вакцинні штами продукували різну кількість екзотоксину. Досліджено вірулентні (*B. anthracis* IBM-92 Z), вакцинні (*B. anthracis* K-79 Z, *B. anthracis* Sterne

34F 2, *B. anthracis* 55, *B. anthracis* СБ, *B. anthracis* Ценковського II) штами, а також сибіркоподібні культури (*B. cereus* 8035, *B. anthracoides* 67, *B. subtilis* ВКМ 17). У процесі експериментальної роботи визначено, що продукція екзотоксину різних штамів збудника сибірки залежить від рН середовища. Встановлено, що при ідентичних показниках рН середовища та умов культивування найвищу продукцію екзотоксину показав вакцинний штам *B. anthracis* К-79 Z. Титр специфічного сибіркового білка становив 1:64. Зміна рН середовища при культивуванні штамів впливає на кількість утворення екзотоксину – основного фактору формування специфічного імунітету проти збудника антракса. Результати досліджень можуть бути застосовані фахівцями ветеринарної служби для відбору продуцентів антигену при розробці нових препаратів проти сибірки в тварин на основі екзотоксинів

Ключові слова: антракс; токсиноутворення; культивування; рН середовища; вірулентні штами; референтні культури