DOI: 10.29326/2304-196X-2023-12-2-171-177

Optimizing a low-temperature preservation technique for *Bacillus anthracis* strains

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SUMMARY

The use of pure microbial cultures is associated with the following key challenges: storage, transportation and resuscitation after a long-term preservation. The currently used anthrax vaccines are produced using various strains of *Bacillus anthracis*. According to the storage passport data, anthrax strains are now stored in 30–40% glycerin solutions, which helps to preserve a sufficient number of viable cells without losses to their pathogenic properties for three years. It is obviously an urgent task to develop a long-term preservation technique for *Bacillus anthracis* strains. The aim of this study was to optimize a low-temperature preservation method for *Bacillus anthracis* strains that ensures viability and no losses to biological properties of the pathogen. Two vaccine strains of *Bacillus anthracis* were selected for the research: i.e. K-STI-79 and 55-VNIIVViM and two cryoprotective media (No. 1 – 15% glycerin solution with 15% glucose solution and No. 2 – 30% neutral glycerin solution in saline solution). At first biological properties of the strains were studied and the number of viable cells was calculated. Later on, the strains were placed into low-temperature preservation facilities, at the temperature of –40 and –70 °C. Six months later, the effect of three thawing cycles on viability and biological properties of the agent was tested: i.e. at room temperature (22 ± 2) °C, in a water bath at a temperature of (37 ± 1) °C and in a household refrigerator at a temperature of (6 ± 2) °C. As demonstrated, the best option is to preserve the cells at –70 °C and thaw them in a water bath at (37 ± 1) °C. Further research will be focused on duration of the low-temperature preservation that will ensure appropriate viability and biological properties of the pathogen.

Keywords: anthrax, *Bacillus anthracis*, strains, low-temperature preservation

Acknowledgment: The work was carried out using the funds of the FSBSI "FCTRBS-ARRVI" within the research "Collecting, maintaining, replenishing and storing strains of highly dangerous diseases (HDD), keeping records of them, studying their biological properties and providing agro-industry companies with HDD strains".

For citation: Rodionov A. P., Artemeva E. A., Melnikova L. A., Sahibullina D. M. Optimizing a low-temperature preservation technique for *Bacillus anthracis* strains. *Veterinary Science Today*. 2023; 12 (2): 171–177. DOI: 10.29326/2304-196X-2023-12-2-171-177.

Conflict of interests: The authors declare that there is no conflict of financial/non-financial interests associated with the paper.

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УДК 619:616.98:579.852.11

Отработка режима низкотемпературной консервации штаммов *Bacillus anthracis*

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РЕЗЮМЕ

Ключевой проблемой использования чистых культур микроорганизмов является их хранение, транспортировка, восстановление жизнеспособности после длительной консервации с сохранением ценных биологических свойств. Применяемые в настоящее время противосибиреязвенные вакцины создаются с использованием различных штаммов *Bacillus anthracis*. На сегодняшний день штаммы возбудителя сибирской язвы, согласно данным паспортов, консервируют в 30–40-процентных растворах глицерина, позволяющих сохранять достаточное количество жизнеспособных клеток, а также свойства возбудителя в течение трех лет. Очевидно, что разработка способа консервации штаммов *Bacillus anthracis* для более продолжительного хранения возбудителя является актуальной задачей. Целью работы было отработать режим низкотемпературной консервации штаммов *Bacillus anthracis*, обеспечивающий сохранность жизнеспособности и биологических свойств возбудителя. Для проведения исследований были отобраны два вакцинных штамма *Bacillus anthracis*: К-СТИ-79 и 55-ВНИИВВиМ, а также две криопротекторные среды: № 1 – 15%-й раствор глицерина с 15%-м раствором глюкозы и № 2 – 30%-й нейтральный раствор глицерина на физиологическом растворе. На первом этапе были изучены биологические свойства штаммов и подсчитано количество жизнеспособных клеток. После чего штаммы были помещены на низкотемпературную консервацию при минус 40 и минус 70 °С. Через 6 месяцев хранения изучали сохранность их жизнеспособности и биологических свойств при трех режимах разморозки: при комнатной температуре (22 ± 2) °С, на водяной бане при температуре (37 ± 1) °С и в бытовом холодильнике при температуре (6 ± 2) °С. Было установлено, что наиболее подходящим режимом явилось хранение клеток при минус 70 °С и размораживание на водяной бане при (37 ± 1) °С. Дальнейшие исследования будут направлены на установление максимально возможной длительности хранения штаммов при низкотемпературном режиме консервации, при которой сохранятся жизнеспособность и биологические свойства возбудителя.

Ключевые слова: сибирская язва, *Bacillus anthracis*, штаммы, низкотемпературная консервация

Благодарность: Работа выполнена за счет средств ФГБНУ «ФЦТРБ-ВНИВИ» в рамках научно-исследовательской работы по теме «Коллекционирование, поддержание, пополнение и хранение штаммов возбудителей особо опасных болезней (ООБ), организация их учета, проведение исследований по изучению биологических свойств и обеспечение предприятий агропромышленного комплекса штаммами возбудителей ООБ».

Для цитирования: Родионов А. П., Артемьева Е. А., Мельникова Л. А., Сахибуллина Д. М. Отработка режима низкотемпературной консервации штаммов *Bacillus anthracis*. *Ветеринария сегодня*. 2023; 12 (2): 171–177. DOI: 10.29326/2304-196X-2023-12-2-171-177.

Конфликт интересов: Авторы заявляют об отсутствии конфликта финансовых/нефинансовых интересов, связанных с написанием статьи.

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INTRODUCTION

Any research into infectious diseases, basic microbiology, structural and molecular biology is based on the use of pure microbial strains that are also crucial for the purposes of biotechnology and biomanufacturing [1, 2]. The use of pure microbial cultures is associated with the following key challenges: storage, transportation and resuscitation after a long-term preservation without any losses to their valuable phenotypic and genotypic characteristics [3, 4].

The Culture Collection Centres that supply research laboratories and biological establishments with valuable pathological strains face an important task to select an appropriate preservation method and adjust it to a particular microorganism [5]. Optimally selected preservation methods and protocols ensure good viability and stable biological properties of specific microbial strains for a long time. Therefore, collection centers use various preservation methods: subculturing, storage in mineral oil, storage in water and water-salt solutions, drying on solid media, low-temperature preservation (from –10 to –80 °C and below), cryopreservation (storage in liquid nitrogen at –196 °C) and freeze-drying [6]. The most frequently used long-term storage lab methods include low-temperature preservation and freeze-drying.

Low-temperature preservation of microorganisms consists in freezing pure cultures of bacteria or viruses in a cryoprotective medium that protects the microorganisms from ice crystals during storage, freezing and thawing [7, 8]. The storage period depends on the storage temperature and the cooling/thawing rate [9]. Many Collection Centers successfully preserve bacterial cultures in modern freezers that maintain temperatures down to –86 °C [6].

Freeze-drying is a desiccation tool used for frozen biomaterials, when water is evaporated in a frozen state (in a vacuum) and does not pass through the liquid state. Thus, the original structure of the freeze-dried subject is retained [7]. Freeze-drying is now recognized as the method ensuring the longest storage of microorganisms, and convenient transportation of the freeze-dried material [10].

Low-temperature preservation is a more available tool, since it does not require a complex infrastructure (except for a freezer), whereas freeze-drying requires more specific equipment and specially trained engineers and technical staff [11]. This method ensures a 10-year storage of microbial cells without any losses to their properties [5].

There are publications in Russian and foreign literature on low-temperature storage technique mainly used for BSL III and IV pathogens [12–15]. However, development of preservation protocols for BSL I and II pathogens is equally important.

Most highly dangerous diseases were eradicated with the help of various immunobiological drugs produced on pure microbial cultures [16–19]. Anthrax is a highly dangerous zooanthroponosis, widely spread in many countries, the spore-forming microorganism *Bacillus anthracis* is the causative agent [20]. Relative freedom of our country from this disease has been achieved by annual vaccination of all the susceptible animals, as well as humans who may face a risk of infection [21]. Animals and humans are immunized with the vaccines produced from *B. anthracis* living cells: strains 55-VNIIVViM and STI-1, correspondingly [22]. In order to ensure biological safety of our country, it is strategically important to preserve those properties of the pathogen that contribute to a strong anti-anthrax immunity.

Currently, anthrax strains are stored in Collection Centers in a freeze-dried form or in sealed ampoules with 30-40% glycerin solutions. Freeze-drying of *B. anthracis* cells enables to store them for decades, however, recent research done by Russian experts demonstrate that freeze-drying of strains of highly dangerous diseases makes it difficult to achieve a required biosafety level in the course of work. This is explained by the fact that freeze-drying is accompanied by generation of aerosols, which contain cells of the freeze-dried pathogen [23]. Preservation of *B. anthracis* in glycerin solutions is a safer method; however, in this case pathogens may be stored maximum for 3 years (according to the strain data sheet). There are no data in Russian or foreign literature on low-temperature preservation of *B. anthracis* strains. However, based on the data available [5, 6, 8], it can be assumed that, in terms of pathogen handling, preservation method is safer than freeze-drying, and it ensures longer pathogen viability than storage in glycerin solutions.

The above data demonstrate how important it is to search for an optimal low-temperature preservation tool for *B. anthracis* strains. The scientific novelty of the research consists in comparing viability of the *B. anthracis* cells preserved and stability of their biological characteristics under different low-temperature preservation conditions, when two cryoprotective media are used.

Based on the above, the purpose of this work was to optimize low-temperature preservation tool for *B. anthracis* strains that will preserve appropriate viability and biological characteristics of the pathogen.

MATERIALS AND METHODS

Strains. Two anthrax strains from the collection were used for the research: K-STI-79 and 55-VNIIVViM, stored in 30% and 40% glycerin, respectively. These two strains were selected for safety reasons, as they are vaccine strains, which retain all the properties of the pathogen, except for the capsule formation.

Nutrient media: meat-peptone agar (MPA), meat-peptone broth (MPB), 5% blood MPA, 12% gelatin, skimmed milk, GKI medium, Hottinger broth produced by the FGBSI "FCTRBS-ARRVI".

Reagents. For the purposes of this work we used non-specific preservative-free horse serum produced by FKP "Kursk Biofactory – BIOK Company" (Russia); gentian violet (p. a.), basic fuchsin (p. a.), Iodine crystals (p. a.), crystalline glucose (p. a.), glycerin (p. a.) produced by OOO NPO "TatHimProdukt" (Russia).

Equipment. The culture was handled in biosafety cabinet BMB-"Laminar-S"-1,2 PROTECT (LAMSYSTEMS CC, Russia). Cultures were grown in a vertical water thermostat TV-40 (Russia). Smears were examined using MICMED-5 microscope ("LOMO" JSC, Russia). A desktop centrifuge OPn-8 (OAO TNK "Dastan", Kyrgyzstan) was used for centrifugation.

Methods. Before low-temperature preservation, we studied biological properties of the strains given in strain data sheets, according to MUC 4.2.2413-08 "Laboratory diagnostics and detection of anthrax causative agent"[1].

After checking the properties, suspensions of spore-shaped cells were prepared in saline solution. The concentration of viable cells in suspensions was determined after inoculation onto MPA followed by CFU counting[2].

Then the prepared suspension was centrifuged and the supernatant was discarded. The precipitate cells were mixed with 1 cm^3 of cryoprotective medium No. 1 (15% glycerin solution with 15% glucose solution) and cryoprotective medium No. 2 (30% neutral glycerin solution in saline solution) and placed in plastic cryotubes with screw caps. After protective media were added, the tubes with the resulting suspension were carefully rotated along the vertical axis, kept for 30 minutes at room temperature to better intermix the medium with the cells and placed into a low-temperature preservation unit (at –40 °C and –70 °C).

For the purposes of comparison, a cell suspension was also prepared, put into 30% and 40% glycerin solutions (K-STI-79 and 55-VNIIVViM, respectively) and stored at 4 °C in accordance with the recommendations of the strain data sheet.

The preserved cells were thawed after a 6-month preservation, until the ice crystals in test tubes completely disappeared. The following different thawing techniques were used:

– at room temperature (22 ± 2) °C;
– in a water bath at a temperature of (37 ± 1) °C;
– in a household refrigerator at a temperature of (6 ± 2) °C.

After thawing, serial 10-fold dilutions were prepared in 0.9% saline solution, then the resulting dilutions were inoculated into Petri dishes and CFU counted. Each sample was inoculated into 5 Petri dishes. The biological properties of the strains were studied as described above.

The Mann – Whitney U test was used to assess the statistical significance of the obtained results. Differences at $p \leq 0.01$ were considered statistically significant (after recalculating the number of comparisons). Quantitative data in Figure 3 and in Table 2 are represented as $M \pm SD$ (where M is the mean value, SD is the standard deviation) [24].

RESULTS AND DISCUSSION

Biological properties of B. anthracis strains before low-temperature preservation. When assessing cultural and morphological properties, it was found that the strains in the MPB demonstrated a growth typical for anthrax, i.e. they look like a lump of cotton wool at the bottom of a transparent medium (Fig. 1A). When shaking, moiré patterns appeared on the surface of the medium. On day five, the bacteria formed a strong chain along the tube walls on the surface of the medium. On MPA, colonies are grey-white; with a ground-glass surface and prominent wisps (Fig. 1B). Under low magnification, the grown colonies had 'curled hair' appearance – R-shape (Fig. 2A). Microscopy of gram-stained smears demonstrated that the strain cells formed chains of large, spore-forming, gram-positive rod-shaped bacteria (Fig. 2B).

The cultures demonstrated a lack of motility, as observed in the motility test. A 24-hour Incubation of cells on 5% blood agar revealed no hemolytic activity. Five days later, a typical strain growth occurs as "inverted fir tree" appearance in 12% gelatin with typical "stocking-like" liquefaction on the surface. The growth cultures cause coagulation and peptonization of skimmed milk. Cells grown in GKI medium with subsequent Rebiger staining demonstrated no ability to form capsules. The "string pearl" appearance test showed that the strains are sensitive to penicillin.

The study of the biological properties of the vaccine strains K-STI-79 and 55-VNIIVViM *B. anthracis* revealed that all the properties corresponded to strain data sheet and, except for capsule formation, are typical for anthrax pathogen (Table 1).

Counting colony-forming units of B. anthracis strains before low-temperature preservation. The next stage was to count CFU for each strain before cryoprotective media were added. For this purpose, cell suspensions were prepared in saline solution. After that, tenfold cell dilutions

[1] MUC 4.2.2413-08 Laboratory diagnosis and detection of anthrax causative agent: methodical instructions. Moscow: Federal Center of Hygiene and Epidemiology of Rospotrebnadzor; 2009. 69 p. Available at: https://files.stroyinf.ru/Data2/1/4293752/4293752010.pdf. (in Russ.)

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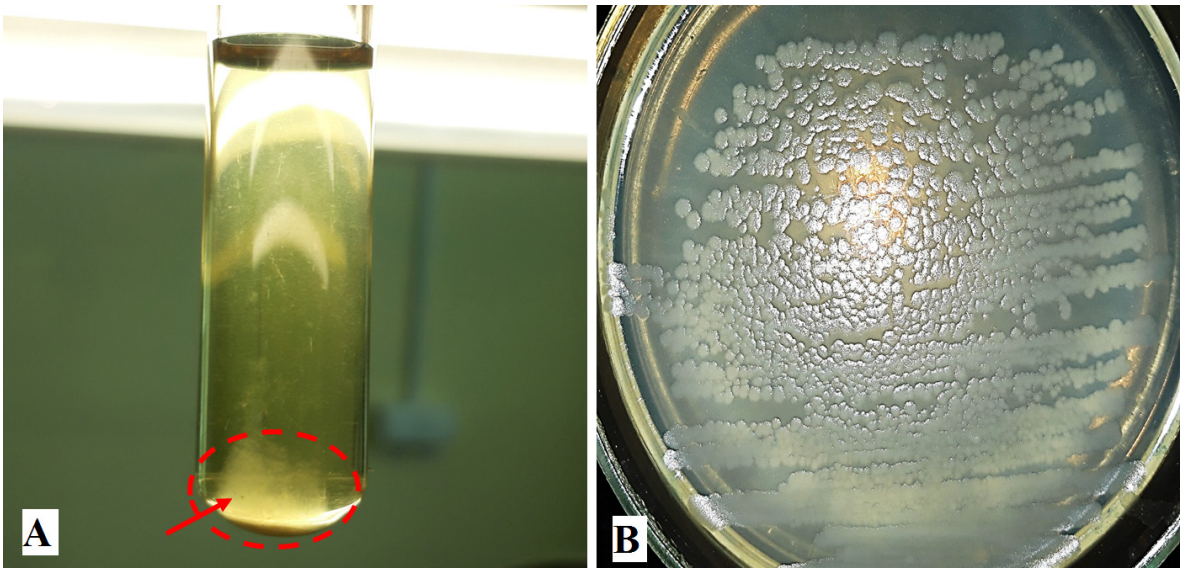

Fig. 1. Culture properties of Bacillus anthracis strain 55-VNIIVViM after 24-hour cultivation:
A – the culture growth looks like a lump of cotton wool (indicated by an arrow) in MPB;
B – the growth of colonies on MPA

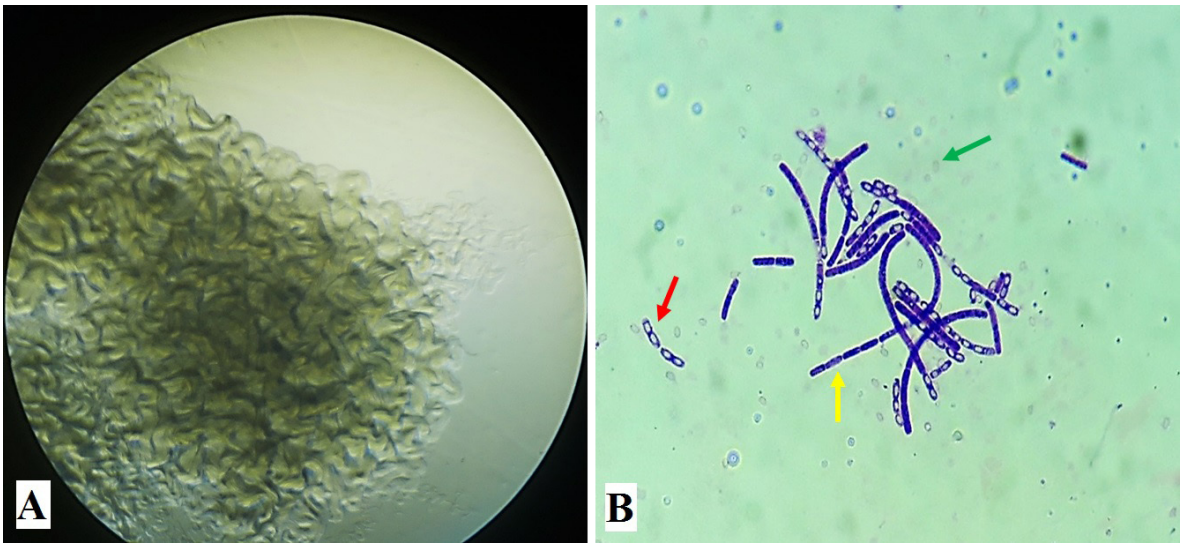

Fig. 2. Colony morphology and shape of B. anthracis bacteria strain 55-VNIIVViM cells after the 24-hour cultivation:
A – R-shaped colonies under low magnification (8 × 40);
B – Gram staining of the strain (yellow arrow – vegetative cell forms; red – emerging spores; green – spores)

were prepared (up to 10^{-5}), inoculated onto Petri dishes with MPA, cultivated at 37 °C for 24 hours and the grown colonies were counted.

The average CFU count for the tested strains is shown in Figure 3.

After the grown colonies were counted, the prepared suspensions were centrifuged at 4,500 rpm for 30 minutes. The precipitated cells were mixed with 1 cm^3 of cryoprotectors, placed for preservation at –40 °C and –70 °C, as well as in 30% and 40% glycerin solutions at (6 ± 2) °C.

Solutions of 15% glycerin with 15% glucose and 30% glycerin were cryoprotective media of choice. The choice of these cryoprotectants resulted from the fact that glycerin is the most widely used protective medium. Glycerol solutions of various concentrations were first used for preservation of pathogenic prokaryotes and viruses as early as the beginning of the 20th century [25]. Currently, it has become the "gold standard" for cryopreservation [26]. As noted by many foreign experts, the glucose solution (1 to 18%) added to the mixture of cryoprotectors improves the survival rate of different microorganisms [8, 25]. Our choice of this combination of solutions as a protective medium was explained by the fact that glucose belongs to protectors that penetrate the cell wall, but do not pass through the cytoplasmic membrane. While glycerin can penetrate through the cytoplasmic membrane of cells [25]. Thus, it can be assumed that the combination of these solutions should ensure greater survival of the cryopreserved cells.

Comparing effectiveness of low-temperature preservation of B. anthracis strains. The work shows that the viability

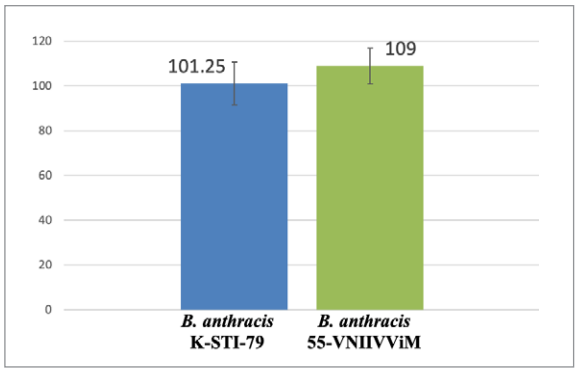

Fig. 3. CFU (M ± SD) of B. anthracis strains K-STI-79 and 55-VNIIVViM before a low-temperature preservation

Table 1
Biological properties of *B. anthracis* vaccine strains K-STI-79 and 55-VNIIVViM

Criterion/property	*B. anthracis* K-STI-79	*B. anthracis* 55-VNIIVViM
Motility	–	–
Hemolytic properties	–	–
Proteolytic properties: 12% gelatin skimmed milk	 + +	 + +
Capsule formation	–	–
Penicillin sensitivity	+	+
Spore formation	+	+

of anthrax strains cells was best preserved when stored at a temperature of –70 °C with further thawing in a 37 °C water bath (Table 2). At the same time, there was no significant difference in viability of cells preserved in different cryoprotectors. The CFU count did not show any statistically significant difference from the CFU count reported during storage in glycerin solutions in a refrigerator. While evaluating the results obtained, it is necessary to take into account the fact that the preparation of cells for low-temperature preservation is associated with huge losses resulting from centrifugation, mixing with a cryoprotector, freezing and thawing, which do not occur during storage in a household refrigerator. It suggests that the selected preservation tool helps to save a larger number of cells than other techniques.

Strain cells stored at –40 °C demonstrated significantly less viability under all thawing schemes. However, thawing at 37 °C helped to save a larger number of viable cells. The results obtained can be explained by the fact that the storage at –40 °C does not help to completely stop recrystallization. Thawing of the stored cells in a refrigerator is a time-consuming process that may result in prolonged ice recrystallization, which is one of the major factors that destroy frozen cells [8, 26].

CFU count after the low-temperature preservation was followed by an assessment of the pathogen biological properties, which demonstrated their full compliance with strain data sheet.

Thus, we can say that the selected method of low-temperature preservation of anthrax pathogen at –70 °C looks promising for further work. Further research will be focused on duration of strain storage using a low-temperature preservation method that will ensure the pathogen viability and preserve its biological properties.

CONCLUSION

The conducted research demonstrate that anthrax cultures stored at a temperature of –40 °C or –70 °C, retained their viability and biological properties for 6 months. Comparison of two preservation methods (at –40 °C and at –70 °C) allowed us to conclude that storage at –70 °C is more preferable.

Table 2
CFU count of *B. anthracis* cells after low temperature preservation for 6 months ($M \pm S_D$)

Cryoprotector	CFU count before freezing	Temperature of storage	CFU count			
			after thawing at a temperature of			after storage in 30/40% glycerin at a temperature of
			(22 ± 2) °C	(37 ± 1) °C	(6 ± 2) °C	(6 ± 2) °C
B. anthracis 55-VNIIVViM						
Medium No. 1	109.00 ± 8.04	–40 °C	93.40 ± 1.81*	94.20 ± 2.58*	87.80 ± 5.71*	101.80 ± 3.96
		–70 °C	100.40 ± 2.96	101.60 ± 3.43	90.80 ± 2.86*	
Medium No. 2		–40 °C	92.20 ± 2.77*	93.80 ± 3.11*	88.40 ± 4.77*	
		–70 °C	101.00 ± 2.12	102.40 ± 2.40	91.40 ± 3.20*	
B. anthracis K-STI-79						
Medium No. 1	101.20 ± 9.50	–40 °C	94.20 ± 4.43	94.60 ± 3.64	88.00 ± 2.73*	101.20 ± 9.55
		–70 °C	100.20 ± 6.26	98.00 ± 4.47	86.40 ± 5.31	
Medium No. 2		–40 °C	92.60 ± 4.82	92.00 ± 1.87*	90.20 ± 3.70	
		–70 °C	101.00 ± 4.30	97.40 ± 5.12	92.80 ± 3.34	

* statistically significant difference ($p \leq 0.01$).

At this stage of research, comparison of pathogen storage in two protective media did not allow us to choose a more effective one. Such comparisons will be made after a longer storage period, and as a result, an optimal cryoprotector will be selected.

Comparison of three thawing methods used for the tested strains revealed that the most gentle way is to thaw the cells in a water bath at +37 °C, because it helps to preserve largest number of viable cells.

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Received 17.02.2023
Revised 20.03.2023
Accepted 30.03.2023

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Сахибуллина Дания Минзагитовна, старший лаборант лаборатории коллекции штаммов микроорганизмов ФГБНУ «ФЦТРБ-ВНИВИ», г. Казань, Россия; *https://orcid.org/0000-0003-2333-9704.*