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OPTIMIZATION OF FREEZING CONDITIONS FOR CLINICAL MICROORGANISM STRAINS DURING LONG-TERM STORAGE AT -20°C

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Abstract. Storage of collection microbial strains at -20°C in the absence of low-temperature equipment is accompanied by cell damage and reduced viability. Objective. To compare the effectiveness of different freezing regimes (including available two-step methods) for preserving clinical strains of 8 microbial species during long-term storage. Materials and Methods. 24 strains (E. coli, S. aureus, S. epidermidis, B. subtilis, B. cereus, K. pneumoniae, P. aeruginosa, C. albicans; 3 strains each) were stored in 20% glycerol at -20°C for 10 months. Four regimes were compared: standard (ST), programmed two-step (PZ-1: +4°C 30 min → -20°C 60 min), rapid transition through -80°C (PZ-2: 60 min in dry ice), shock freezing (ShZ: 24 h at -80°C). CFU/mL was assessed monthly. Results. PZ-2 and ShZ provided the greatest protective effect for Gram-negative bacteria: after 6 months for E. coli – 5.5±0.4 and 5.6±0.4 lg CFU/mL vs. 4.8±0.6 lg with ST ($p<0.01$); for K. pneumoniae – 5.2-5.3 lg vs. 4.5±0.7 lg ($p<0.01$). For spore-forming bacteria and C. albicans, the regime had no effect ($p>0.05$). Conclusions. Two-step freezing with preliminary cooling to -80°C is recommended, increasing the viability of sensitive strains by 0.5-0.8 lg CFU/mL.

Keywords: cryopreservation, freezing regimes, -20°C, clinical strains, viability, Gram-negative bacteria.

-20°C HARORATDA UZOQ MUDDAT SAQLASHDA MIKROORGANIZMLARNING KLINIK SHTAMMLARINI MUZ LATISH REJIMLARINI OPTIMALLASHTIRISH

Dolzarbligi. Past haroratli uskunalar bo‘lmagan sharoitda mikroorganizmlarning kolleksion shtammlarini -20°C haroratda saqlash hujayralarning shikastlanishi va ularning hayotchanligini

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pasayishi bilan kechadi. Tadqiqot maqsadi. Uzoq muddat saqlashda 8 turdagi mikroorganizmlarning klinik shtammlarini saqlab qolish uchun turli xil muzlatish rejimlarining (shu jumladan, mavjud ikki bosqichli usullar) samaradorligini solishtirish. Materiallar va usullar. 24 ta shtamm (*E. coli, S. aureus, S. epidermidis, B. subtilis, B. cereus, K. pneumoniae, P. aeruginosa, C. albicans;* har bir turdan 3 tadan) 20% glitserin muhitida -20°C haroratda 10 oy davomida saqlandi. To‘rt rejim solishtirildi: standart (ST), dasturli ikki bosqichli (PZ-1: +4°C 30 min → -20°C 60 min), -80°C orqali tez o‘tish (PZ-2: quruq muzda 60 min), shokli muzlatish (ShZ: -80°C da 24 soat). Har oyda KOE/ml baholandi. Natijalar. Gram-manfiy bakteriyalar uchun eng katta himoya ta’sirini PZ-2 va ShZ ta’minladi: 6 oydan keyin E. coli uchun – 5,5±0,4 va 5,6±0,4 lg KOE/ml (ST da 4,8±0,6 lg ga nisbatan, p<0,01); K. pneumoniae uchun – 5,2-5,3 lg (4,5±0,7 lg ga nisbatan, p<0,01). Spora hosil qiluvchi bakteriyalar va C. albicans uchun rejim ta’sir qilmadi (p>0,05). Xulosalar. -80°C gacha oldindan sovutish bilan ikki bosqichli muzlatish tavsiya etiladi, bu sezgir shtammlarning saqlanishini 0,5-0,8 lg KOE/ml ga oshiradi.

Kalit so'zlar: kriokonservatsiya, muzlatish rejimlari, -20°C, klinik shtammlar, hayotchanlik, Gram-manfiy bakteriyalar.

Аннотация. Актуальность. Хранение коллекционных штаммов микроорганизмов при температуре -20°C в условиях отсутствия низкотемпературного оборудования сопровождается повреждением клеток и снижением их жизнеспособности. Цель исследования – сравнить эффективность различных режимов замораживания (включая доступные двухэтапные методы) для сохранения клинических штаммов 8 видов микроорганизмов при длительном хранении. Материалы и методы. 24 штамма (E. coli, S. aureus, S. epidermidis, B. subtilis, B. cereus, K. pneumoniae, P. aeruginosa, C. albicans; по 3 штамма каждого вида) хранили в 20% глицерине при -20°C в течение 10 месяцев. Сравнивали 4 режима: стандартный (СТ), программируемый двухэтапный (ПЗ-1: +4°C 30 мин → -20°C 60 мин), быстрый переход через -80°C (ПЗ-2: 60 мин в сухом льду), шоковую заморозку (ШЗ: 24 ч при -80°C). Ежемесячно оценивали КОЕ/мл. Результаты. Наибольший защитный эффект для грамотрицательных бактерий обеспечивали ПЗ-2 и ШЗ: через 6 мес для E. coli – 5,5±0,4 и 5,6±0,4 lg КОЕ/мл против 4,8±0,6 lg при СТ (p<0,01); для K. pneumoniae – 5,2-5,3 lg против 4,5±0,7 lg (p<0,01). Для спорообразующих бактерий и C. albicans режим не влиял (p>0,05). Выводы. Рекомендовано двухэтапное замораживание с предварительным охлаждением до -80°C, что повышает сохранность чувствительных штаммов на 0,5-0,8 lg КОЕ/мл.

Ключевые слова: криоконсервация, режимы замораживания, -20°C, клинические штаммы, жизнеспособность, грамотрицательные бактерии.

Relevance. Preservation of collection strains of microorganisms is a fundamental task for ensuring the quality of scientific research, educational process and diagnostic work. Cryopreservation at ultra-low temperatures (-70 °C and below) using programmable freezing is considered the optimal method of long-term storage [1]. However, in conditions of limited logistical support for many microbiological laboratories and departments, it becomes necessary to use affordable storage methods at -20 ° C. According to literature data, storage at -20 ° C is accompanied by a complex of damaging factors: the formation of intracellular ice crystals that damage membrane structures, increased osmotic pressure, changes in the pH of the medium and concentration gradients [2]. Gram-negative bacteria are particularly sensitive to such conditions,

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which requires the development of optimal freezing regimes available for routine laboratory practice.

Literature review. Gracheva et al. The review of traditional and new protective media highlights the need to test recommended media for specific bacterial species and the storage temperature used [3]. The authors note that one of the areas of improvement of low-temperature conservation technologies is the search for natural protectors that ensure the survival of bacteria under stressful conditions. Smirnova et al. The mechanisms of the protective effect of cryoprotectors on *E. coli* were investigated. coli and showed that cell permeability, estimated by the release of low molecular weight compounds into the medium, is significantly less impaired when using dextran compared with glycerin [2]. The authors also found that dextran-protected cells are resistant to detergent-induced lysis after freezing to -10°C. Hubálek [4], in a fundamental review of cryoprotectors used in cryopreservation of microorganisms, systematized data on the effectiveness of various protective media. The author points out that dimethyl sulfoxide (DMSO), methanol, ethylene glycol, propylene glycol and blood serum are among the most successful cryoprotectors, while glycerin, polyethylene glycol, PVP and sucrose are less effective. However, the author emphasizes that the optimal cryoprotector for a particular microorganism should be selected empirically. Research by Vysekantsev et al. [5] showed that when cooled to -20 °C, -40 °C, -75 °C and subsequent storage, immobilized microorganisms in an alginate gel did not die, however, at -20 °C, -40 °C, -75 °C in most samples, cell death began between the 1st and 3rd months of storage. Lactobacilli were the most resistant to storage at these temperatures, and S. Cerevisiae yeast was the most sensitive [6].

The purpose of this study is to compare the effectiveness of various freezing regimes (including available two–stage methods without special equipment) for preserving clinical strains of 8 species of microorganisms during long-term storage at -20 °C.

Materials and methods. Objects of research. The study included 24 strains of microorganisms from the bacteriological laboratory of the TMU clinic and the laboratory of the Department of Microbiology, Virology and Immunology of TMU (cultures were obtained earlier upon official request from the Russian National Research Center for Biological Research) represented by 8 species:

- *Escherichia coli* (3 strains, including 1 standard ATCC 25922)
- *Staphylococcus aureus* (3 strains, including 1 standard ATCC 29213)
- *Staphylococcus epidermidis* (3 strains, including 1 standard ATCC 14990)
- *Bacillus subtilis* (3 clinical strains)
- *Bacillus cereus* (3 clinical strains)
- *Klebsiella pneumoniae* (3 clinical strains)
- *Pseudomonas aeruginosa* (3 strains, including 1 standard ATCC 27853)
- ***Candida albicans* (3 clinical strains)**

Storage conditions. All microorganisms were stored in a 20% glycerol solution (glycerol medium) at -20 °C for 10 months (May 2025 – March 2026). 4 freezing modes were compared:

1. Standard (CD) – direct placement of test tubes in the freezer -20 ° C.
2. Programmable two-stage non–hardware method (PZ-1) - 30 min at +4 ° C, then 60 min at -20 ° C, followed by transfer to -20 °C for storage (adapted from the two-stage cooling method described by Hubálek).
3. Fast transition through -80 ° C (PZ-2) – 60 min in a thermos with dry ice (-78 ° C) followed by transfer to -20 ° C (modification of the Vysekantsev et al. method).
4. Shock freezing (SHF) – pre-storage for 24 hours at -80 ° C (in in a low-temperature refrigerator), followed by transfer to -20 ° C for long-term storage.

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Methods for assessing viability. The strains were verified monthly, assessing viability by counting colony-forming units (CFU/ml) after sowing on dense nutrient media. Used:

- Blood agar (for all types)
- The Endo Environment\ Poppy seeds (for enterobacteria)
- Saburo environment (for C. albicans)

The crops were incubated at 37 ° C for 24-48 hours (for C. albicans – 48-72 hours). The colonies were counted in three repetitions for each sample.

Statistical processing. Statistical processing was performed using the StatTech v.4.0 software package (Russia) and SPSS Statistics 26.0 (IBM, USA). The average values (M) and standard deviations (SD) were calculated. To determine the significance of the differences, the Student's t-test for independent samples and two-factor analysis of variance (ANOVA) were used. The differences were considered significant at $p<0.05$.

Results. Table 1 shows data on the viability of microorganisms after 6 months of storage under various freezing conditions.

Table 1. Viability of microorganisms (lg CFU/ml, M±SD) after 6 months of storage under various freezing conditions (medium – 20% glycerin)

The microorganism	Initially (0 month)	DC	PZ-1	PZ-2	ShF
B. subtilis	6,0±0,2	5,8±0,3	5,8±0,3	5,9±0,2	5,9±0,2
B. cereus	6,0±0,2	5,7±0,4	5,7±0,3	5,8±0,3	5,8±0,3
S. aureus	6,0±0,2	5,5±0,4	5,6±0,3	5,8±0,3*	5,8±0,3*
S. epidermidis	6,0±0,2	5,6±0,4	5,7±0,3	5,9±0,2*	5,9±0,2*
E. coli	6,0±0,2	4,8±0,6	5,1±0,5	5,5±0,4**	5,6±0,4**
K. pneumoniae	6,0±0,2	4,5±0,7	4,8±0,6	5,2±0,5**	5,3±0,5**
P. aeruginosa	6,0±0,2	4,7±0,7	5,0±0,6	5,3±0,5**	5,4±0,5**
C. albicans	6,0±0,2	5,8±0,3	5,8±0,3	5,9±0,2	5,9±0,2

*Note: * – differences with the control (CT) are significant at $p<0.05$; ** – $p<0.01$*

As can be seen from Table 1, the greatest protective effect for gram-negative bacteria was provided by the PZ-2 and SHF modes. The viability of E. coli after 6 months under these regimes was 5.5±0.4 and 5.6±0.4 lg CFU/ml, respectively, versus 4.8±0.6 lg under the standard regime ($p<0.01$). For *K. pneumoniae*, an increase was noted from 4.5±0.7 to 5.2-5.3 lg CFU/ml ($p<0.01$), for *P. aeruginosa* – from 4.7±0.7 to 5.3-5.4 lg CFU/ml ($p<0.01$). For gram–positive cocci, the effect was less pronounced, but also significant: S. aureus - 5.8±0.3 lg with PD-2 and SD versus 5.5±0.4 lg with CD ($p<0.05$). For spore-forming bacteria (*B. subtilis, B. cereus*) and *C.*

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albicans, the freezing regime had no statistically significant effect ($p>0.05$): viability after 6 months was 5.8-5.9 lg CFU/ml under all regimes.

Two-factor analysis of variance confirmed the significant influence of the factors "type of microorganism" ($F=28.3$; $p<0.001$), "freezing mode" ($F=22.9$; $p<0.001$) and their interactions ($F=2.0$; $p<0.01$).

Discussion. The results obtained are consistent with the data of the world literature and expand the understanding of the possibilities of storing microorganisms at -20 ° C. According to the medical encyclopedia, the optimal regime is a slow decrease in temperature to -20 ° C and a rapid decrease thereafter [1]. Our data confirm that two-stage modes (PZ-1, PZ-2) and shock freezing (SHF) are more effective than a standard single-stage room at -20 °C, especially for gram-negative bacteria. This is due to a decrease in damage from intracellular crystallization during rapid passage of the critical temperature range [2]. Research by Smirnova et al. It has been shown that E. coli cells protected by non-penetrating cryoprotectors (dextran) are resistant to lysis after freezing to -10°C -2. In our study, the fast-freezing regimes (PP-2 and SHF) also provided better preservation of E. coli, which may be due to the formation of smaller ice crystals and less damage to the membranes. Gracheva et al. The review of traditional and new protective media highlights the need to test media for specific bacterial species and highlights the promise of natural protectors. Our data confirm that species specificity is a key factor: for Gram-negative bacteria, the freezing regime is critically important, whereas bacilli and Candida albicans are resistant to a wide range of conditions. Vysekantsev et al. It was found that when stored at -20 °C, cell death begins between the 1st and 3rd months. In our study, with the standard mode (CD), the critical reduction for *K. pneumoniae* was already observed by the 3rd month (4.5±0.7 lg), whereas with optimized modes (PZ-2, SHF), safety remained acceptable up to 6 months. Hubálek points out in his review that DMSO is considered the most versatile cryoprotector, but glycerin is less effective but less toxic. Our results show that using 20% glycerin in combination with optimized freezing modes can achieve acceptable preservation even for sensitive gram-negative bacteria.

Conclusions

1. The greatest protective effect for gram-negative bacteria (*E. coli, K. pneumoniae, P. aeruginosa*) when stored in 20% glycerin at -20°C provides modes with a rapid transition through -80 °C (PZ-2) and shock freezing (SHF), increasing viability by 0.5-0.8 lg CFU/ml compared to the standard mode ($p<0.01$).

2. For spore-forming bacteria (*B. subtilis, B. cereus*) and *C. albicans*, the freezing mode It has no statistically significant effect ($p>0.05$) – these microorganisms can be stored under any freezing regime.

3. Two-factor analysis of variance revealed a significant influence of the factors "species" ($p<0.001$), "mode" ($p<0.001$) and their interactions ($p<0.01$), which confirms the species specificity of optimal cryopreservation conditions.

4. In the absence of low-temperature equipment (-80°C) two-stage freezing using dry ice (PZ-2) or the fastest possible cooling in a metal tripod at -25...-30 ° C is recommended, which is available for routine laboratory practice.

References

1. Замораживание // Медицинская энциклопедия. – Режим доступа: https://www.med-enc.narod.ru/sprav/090.htm

AMERICAN ACADEMIC PUBLISHER
INTERNATIONAL JOURNAL OF MEDICAL SCIENCES
ISSN NUMBER: 2692 - 5206 Volume 6. No 03. March ,2026

2. Smirnova L.F., et al. Mechanisms of the protective action of cryoprotectors on Escherichia coli cells // Mikrobiologiia. – 1988. – Vol. 57, No. 3. – P. 494-498.

3. Грачева И.В., Валова Т.В., Григорьева Г.В. Традиционные и новые защитные среды для низкотемпературной консервации бактерий // Проблемы особо опасных инфекций. – 2011. – № 4(110). – С. 36-40.

4. Hubálek Z. Protectants used in the cryopreservation of microorganisms // Cryobiology. – 2003. – Vol. 46, No. 3. – P. 205-229.

5. Vysekantsev I.P., Petrov I.V., Buriak I.A., Martsenyuk V.P. Viability of probiotic strains of microorganisms and their adhesion to human erythrocytes after immobilization in alginate gel and storage at different low temperatures // Актуальні проблеми сучасної медицини. – 2020. – № 4. – С. 115-124.

6. Jang I.H., Lee A.R., Song I.G., Kim S.K. Optimization of cryoprotectants for improved survival rates of animal probiotics Lactobacillus plantarum, Bacillus subtilis, and Saccharomyces cerevisiae // Korean Journal of Microbiology. – 2024. – Vol. 60, No. 3. – P. 130-137.