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Improved production strain maintenance technique for *Burkholderia mallei* 5584 (Master seed) used for glander diagnostic agent production

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ABSTRACT

One of the aspects important for strain collection maintenance is the optimization of existing methods and development of new techniques for microbial strain preservation, that is why the improvement of previously developed methods for authentic strain preservation is an urgent task. The article provides information on the maintenance of *Burkholderia mallei* 5584 (Master seed) using previously developed technique, which was supplemented with new stages in accordance with modern requirements for strain collections of highly dangerous disease agents. The previous strain maintenance technique involved its storage in its native state, which facilitated accumulation of genetic mutations and, ultimately modification of bacterial cell properties. To extend the storage time of this strain and to ensure the stability of its biological properties, the freeze-drying method was used. Skimmed milk was used as a cryoprotectant. Freeze-drying was performed under selected conditions. This technique allows for the strain sub-culturing on sensitive models once every 5 years, which is more expedient and safe from an economic and biological point of view. For safe handling of *Burkholderia mallei* 5584 production strain, an inactivation technique using gamma rays at 30 kGy was developed, which allowed to achieve microbial suspension sterility and preserve the bacterial cell structure. When comparing the previously developed and supplemented techniques, it was found that the improved technique of *Burkholderia mallei* 5584 (Master seed) maintenance makes it possible to avoid the loss of its biological properties needed for the production of high-quality laboratory diagnostic agents used for timely disease detection in susceptible animals by diagnostic tests.

Keywords: glanders, *Burkholderia mallei*, passage, freeze-drying, biological properties, gamma irradiation

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Усовершенствование системы поддержания производственного штамма *Burkholderia mallei* 5584 (Master seed), применяемого для изготовления сапных диагностикумов

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РЕЗЮМЕ

Одним из направлений коллекционной деятельности является оптимизация существующих методов и разработка новых технологий консервации штаммов микроорганизмов, поэтому проведение работ по усовершенствованию ранее разработанных методик сохранения аутентичности штаммов

является актуальной задачей. В статье приведена информация по поддержанию производственного штамма *Burkholderia mallei* 5584 (Master seed) с использованием разработанной ранее системы, которая была дополнена новыми этапами согласно современным требованиям, предъявляемым к коллекционным фондам штаммов возбудителей особо опасных болезней. Предыдущая схема поддержания штамма предусматривала его хранение в нативном виде, что способствовало накоплению генетических мутаций и, как следствие, изменению свойств бактериальной клетки. Для увеличения сроков хранения данного штамма и обеспечения стабильности его биологических свойств применен метод лиофилизации. В качестве криопротектора использовали обезжиренное молоко. Сублимационную сушку проводили по выбранному режиму. Данный метод дает возможность пассировать штамм на чувствительных моделях один раз в 5 лет, что более выгодно и безопасно с экономической и биологической точек зрения. Для безопасной работы с производственным штаммом 5584 возбудителя сапа разработан метод его инактивации гамма-лучами при 30 кГр, который позволил добиться стерильности микробной взвеси и сохранить структуру бактериальных клеток. При сравнении ранее разработанной и дополненной схем установлено, что усовершенствованная система поддержания штамма *Burkholderia mallei* 5584 (Master seed) позволяет исключить утрату его биологических свойств, необходимых для производства качественных сапных диагностикумов, используемых для своевременного выявления заболевания у восприимчивых животных при проведении диагностических исследований.

Ключевые слова: сап, *Burkholderia mallei*, пассаж, лиофилизизация, биологические свойства, гамма-облучение

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INTRODUCTION

Freedom from contagious and highly dangerous infectious diseases, in particular from glanders, is ensured by the veterinary service, responsible for anti-epidemic measures aimed at prevention of the pathogen introduction into the Russian Federation using systematic monitoring of the animal health status among horse populations (donkeys, mules), as well as containment and eradication of the disease in case of its occurrence [1].

Glanders is an infectious disease of equine animals, caused by the bacterium *Burkholderia mallei*, which often develops chronic infection. Under natural conditions, feline predators can also be infected (when eating meat of glanderous animals). The infection can be also transmitted to camels and humans [2, 3, 4, 5]. This pathogen is classified as pathogenicity group II (hazard) agent. No specific preventive or therapeutic agents against glanders have been developed yet , and therefore the disease is an exceptional issue for the biological safety of the Russian Federation [6, 7].

Since the late 50s of the last century to the present, glanders has not been reported in Russia. Although there have been cases of glanders suspicion during this period, none of them have been confirmed [8]. To date, a glanders outbreak was reported in the Zabaikalsky Krai followed by the introduction of quarantine (Resolution of the Zabaikalsky Krai Governor of 18.02.2023 No. 8.[1]). The risk of occurrence of new glanders cases can not be excluded due to the infection presence in the countries bordering Russia (Mongolia and China), where the veterinary legal requirements are often not observed, in particular, illegal livestock exchange/movement and import of animal raw materials can occur [1, 9]. Besides modern international contacts, involving glanders-susceptible animals (trade, tours of circuses and animal theaters, equestrian competitions, international auctions, etc.), can create an unpredictable glanders situation.

Currently, glanders is often reported from Mongolia, Turkey, Iran, Iraq, the Arabian Peninsula countries, Brazil, China, India, and the Philippines [9, 10, 11, 12, 13, 14]. According to the World Organization for Animal Health, the Food and Agriculture Organization of the United Nations, and the World Health Organization, there is a tendency in the world of increased glanders cases in humans and animals, which qualifies it as a re-emerging infection.

To prevent the occurrence, importation and introduction of this disease into the country, diagnostic measures are taken using glanders diagnostic agents produced by the Kursk Biofactory – BIOK company (glanders positive serum for complement fixation test – CFT; *B. mallei* antigen for CFT; mallein and *B. mallei* colored antigen for plate agglutination test – AT) using production strain *Burkholderia mallei* 5584. It is provided by the State Strain Collection, containing pathogens of highly dangerous diseases used in the veterinary medicine and livestock industry, where the strain is stored in native and freeze-dried states with its biological properties maintained. A number of authors proved that the storage of microorganisms in their native state does not satisfy the current standards, since mutations accumulate during the sub-culturing process

[1] On the establishment of restrictive measures (quarantine) in the territory of Chita city: Resolution of the Zabaikalsky Krai Governor of 18.02.2023 No. 8. https://media.75.ru/documents/152305/8-ot-18-02-2023.pdf

ultimately resulting in modification of their original biological properties [15, 16, 17, 18]. Taking into account, that one of the aspects important for strain collection maintenance is the optimization of existing methods and development of new techniques for microbial strain preservation, the improvement of previously developed methods for authentic strain preservation is an urgent task [19, 20, 21, 22].

Based on the above, the aim of the work was to improve the production strain maintenance technique for *Burkholderia mallei* 5584 (Master seed), used to preserve its viability and biological properties.

MATERIALS AND METHODS

Activities associated with the maintenance of the *Burkholderia mallei* 5584 (Master seed) were performed in the "State Microbial Collection" of the Federal Center for Toxicological, Radiation and Biological Safety.

The strain is stored in its native form on beef-extract glycerol agar and is subcultured every 30 days followed by testing of its biological properties once a year. The strain stored in freeze-dried state (skimmed milk cryoprotectant) is tested for its viability and compliance with the properties stated in its accession form every 5 years and *in vivo* passaging using sensitive models (golden hamsters) once a year.

The growth properties and cell morphology of the strain stored in native and freeze-dried forms were studied in second-generation cultures grown on beef-extract glycerol agar, beef-extract glycerol broth and Pavlovsky potato agar. The tinctorial properties were studied by microscopy of Gram-stained smears; motility was evaluated by the hanging drop method using light microscopy. The fermentative activities were tested by inoculation in Hiss' medium and skimmed milk, as well as by the formation of hydrogen sulfide in beef-extract glycerol agar and indole in Strogov growth medium inoculated with culture, using test papers (lead acetate papers and oxalic acid papers, respectively), placed above the surface of the medium. The catalase test was performed by adding 3% hydrogen peroxide to the grown culture[2].

The strain pathogenicity was evaluated by inoculation of sensitive laboratory animals (golden hamsters), followed by an assessment of glanders consistent clinical signs, post-mortem lesions in lungs, liver, and spleen and isolation of the pathogen pure culture.

In order to study the agglutination properties of the *Burkholderia mallei* 5584 (Master seed), an antigen and antiserum were prepared, which were tested by plate AT. Serum was prepared by triple immunization of rabbits with inactivated *Burkholderia mallei* 5584 (Master seed) cells at a concentration of 2×10^9 microbial cells/cm^3 by injection into the marginal ear vein at three-day intervals: the first injection was 0.5 cm^3, the second injection was 1.0 cm^3, the third injection was 2.0 cm^3. Total exsanguination of rabbits was performed on day 7 post last injection.

The serum was tested for its antigenic properties (using plate and tube AT and tube CFT).

To determine the specificity, closely related *B. pseudomallei* and *Alcaligenes faecalis* were used [23]. The strains were inoculated on beef broth glycerol agar and meat-peptone agar, incubated at 37 °C for 48 hours, washed with 0.85% NaCl solution and tested by AT and CFT in accordance with SanPiN 3.3686-21[3].

The production strain biological properties were maintained in accordance with the accession form by passaging in golden hamsters, infected subcutaneously in the occipital area.

The experiments with the laboratory animals were performed in accordance with the general ethical principles of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, March 18, 1986). The experiments were scientifically substantiated and approved by the Bioethics Commission of the Federal Center for Toxicological, Radiation and Biological Safety (Protocol No. 10 of 11.09.2023).

To obtain the antigen, the *Burkholderia mallei* 5584 (Master seed) was inactivated with gamma rays using "Issledovatel" device (Russia) at previously determined radiation dose of 30 kGy in the radiobiology department[4].

When optimizing the inactivation modes, doses of 15, 20, 25, 30, 35 kGy were used. For this purpose, cell suspensions with concentrations of 10^9 microbial cells/cm^3 were prepared using sterile 0.85% NaCl solution. The inactivation was tested by inoculation of cells on growth media (beef-extract glycerol agar and broth) followed by incubation for 10 days at 37 °C.

The freeze-drying conditions were optimized using LZ-9 freeze-dryer (Frigera, Czech Republic). Skimmed milk and sucrose-gelatin medium were used as cryoprotectants.

In this work, the technique of the production strain maintenance for the *Burkholderia mallei* 5584 (Master seed) was used [22], which was supplemented with new stages that meet modern requirements for the storage of collection strains.

RESULTS AND DISCUSSION

To improve the quality and conformity of production, reference and vaccine strains, the comprehensive study of the stability of their biological properties is needed with the focus on their characteristic genetically fixed features: morphological, biochemical, antigenic and others [17, 24, 25]. The viability and biological properties of the *Burkholderia mallei* 5584 (Master seed) were studied using the previously developed and supplemented maintenance technique for the strain used for the production of diagnostic agents, in accordance with the modern requirements for the microorganism storage [8, 22, 26].

The obtained results showed that the strain grew on beef-extract glycerol agar in the form of translucent smooth shiny colonies, becoming confluent on day 3–5 after inoculation on the medium surface (Fig. 1A).

[2] Glanders laboratory diagnosis: guidelines. Moscow: Federal Center for Hygiene and Epidemiology of Rospotrebnadzor; 2011. 22 p.

[3] SanPiN 3.3686-21 Sanitary and epidemiological requirements for the prevention of infectious diseases: approved by Resolution of the Chief Sanitary Inspector of the Russian Federation No. 4 on 28.01.2021. https://docs.cntd.ru/document/573660140 (date of access 15.11.2022).

[4] Shashkarov V. P., Gainutdinov T. R., Idrisov A. M., Guryanova V. A., Vagin K. N., Vasilevsky N. M., etc. Methodological recommendations on the use of ionizing radiation for inactivation of livestock infectious disease pathogens. Kazan: MeDDoK; 2021. 17 p. https://doi.org/10.31016/fctrb-viev-2020-2

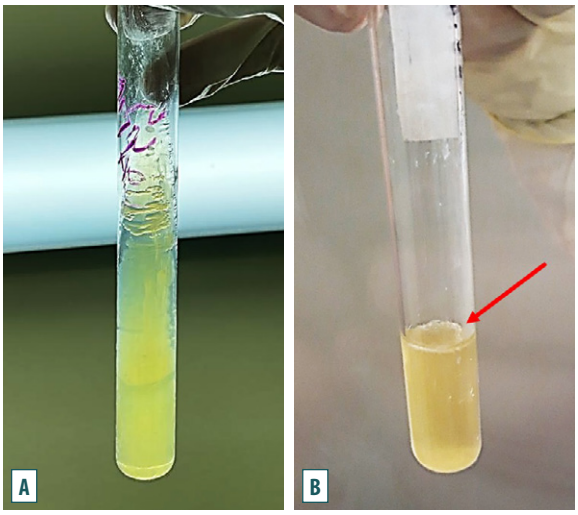

Fig. 1. Growth of Burkholderia mallei strain 5584 (Master seed) on beef-extract glycerol agar (A) and beef-extract glycerol broth (B). The ring and pellicle are indicated with a red arrow on the surface of the growth medium

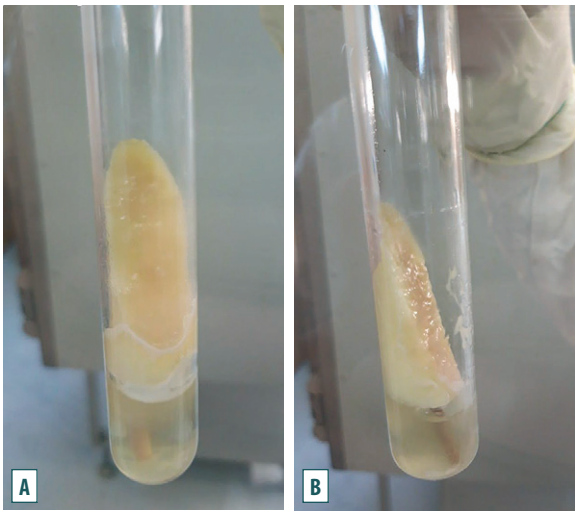

Fig. 2. Growth of Burkholderia mallei 5584 (Master seed) on Pavlovsky potato agar: 2-day culture (A); 5-day culture (B)

The bacterial culture on beef peptone glycerole broth on day 3–5 caused turbidity of the medium, a pellicle and a ring formed on its surface (Fig. 1B). A viscous precipitate was observed at the tube bottom, spinning and breaking when shaken.

When Pavlovsky potato agar was used, amber honey-like growth appeared on day 2 (Fig. 2A), mucous growth of a darker color appeared on day 5 (Fig. 2B).

In the hanging drop, the cells were immotile, but Brownian motion was observed. A smear of the *Burkholderia mallei* 5584 (Master seed) prepared from a 2-day culture and Gram-stained, looked like granular round-end rods when studied by microscopy with oil immersion. The bacteria were gram-negative, colored pink, and caused milk to coagulate without further peptonization (Fig. 3A). During the growth, the test strips changed their color in the broth which suggested the formation of hydrogen sulfide by bacteria (Fig. 3B); 12% gelatin was not liquefied (Fig. 3C), indole and catalase were not formed (the color of the test strip did not change).

The culture of the *Burkholderia mallei* 5584 (Master seed) did not change the color of the Hiss' medium to yellow and did not cause the formation of gas bubbles in floats, since it did not ferment sugars.

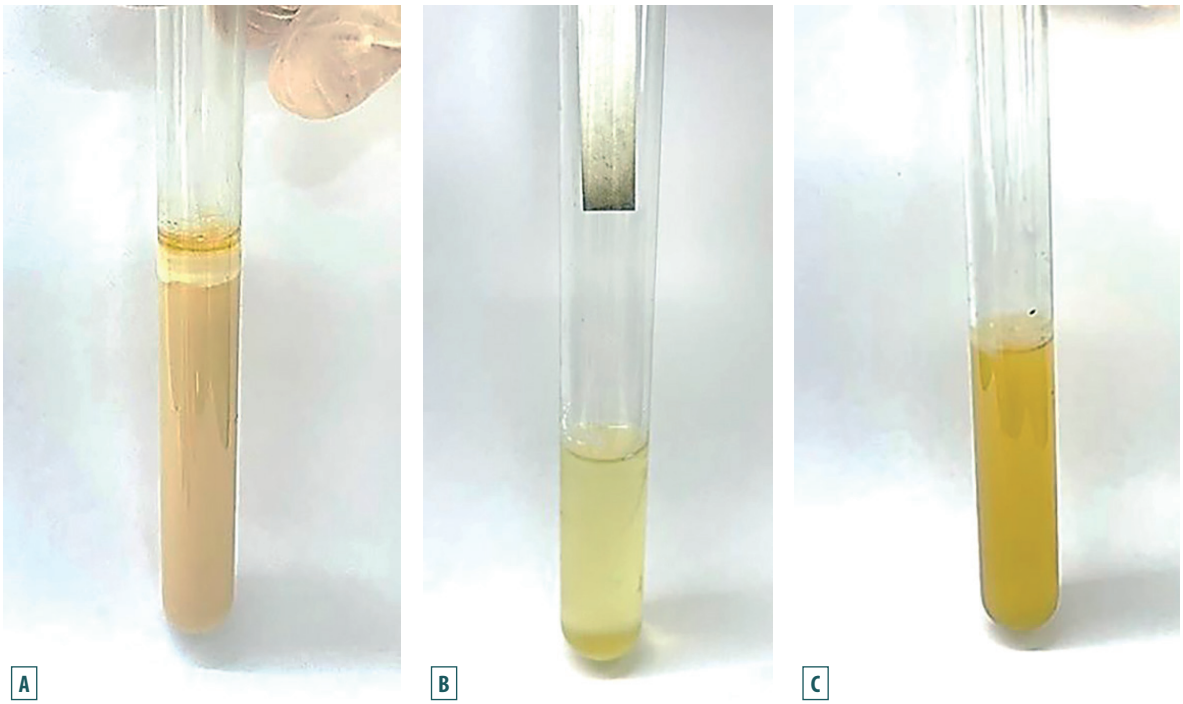

Fig. 3. Milk coagulation without further peptonization (A); formation of hydrogen sulfide (B); no liquefaction of 12% gelatin (C) by Burkholderia mallei 5584 (Master seed)

For safe handling of pathogens of I–II pathogenicity groups (hazard), in particular when preparing antigens and hyperimmune sera, the pathogens must be inactivated by various methods (physical, mechanical, chemical, etc.). The previous technique of strain maintenance included inactivation of cells by autoclaving at a temperature of 120 °C for 15 minutes, which resulted in their complete destruction. Therefore, a gamma radiation dose was selected for the *Burkholderia mallei* 5584 (Master seed). The results are given in Table 1.

It was found that 30 and 35 kGy irradiation completely killed the strain; therefore, a dose of 30 kGy was used in further work.

Plate agglutination test for the agglutination properties of the glanders serum obtained against gamma-irradiated antigen showed positive reaction in the form of small-grained agglutinate formed within 1–2 minutes.

The pathogenicity of the strain was tested by infection of golden hamsters by injecting a 2-day culture grown on beef-extract glycerol agar at 37 °C for 48 hours, washed off with saline solution at a dose of 1×10^9 microbial cells/cm^3 subcutaneously into the occipital area. Animals died on day 5–10. The autopsy revealed the following glanders-consistent post-mortem lesions: a purulent necrotic lesion at the injection site, multiple necrotic 2–3 mm nodules in internal organs (liver, spleen, lungs). A pure bacterial culture was isolated from inoculations made from internal organs, heart blood and injection site.

Table 1
Determination of *Burkholderia mallei* 5584 (Master seed) inactivation dose by gamma irradiation

No.	Gamma radiation dose, kGy	Antigen inactivation
1	15	–
2	20	–
3	25	–
4	30	+
5	35	+

Testing of the antigenic properties of the *Burkholderia mallei* 5584 (Master seed) using sera from inoculated laboratory animals showed that tube AT titre was 1:1600, CFT titre – 1:320, plate AT titre – 1:120.

The resulting serum gives cross-reactions in AT and CFT with *B. pseudomallei* – 1:1600 and *Alcaligenes faecalis* – 1:40.

In order to preserve the purity of unique production and reference microorganism strains, they are passaged *in vivo* using sensitive laboratory animals [15, 27, 28, 29]. In this regard, in order to maintain the biological properties of the production strain *Burkholderia mallei* 5584 (Master seed), as stated in its accession form, it was passaged in golden hamsters. At the same time, a pure strain

Table 2
Original and supplemented techniques of *Burkholderia mallei* 5584 (Master seed) maintenance

No.	Initial technique	Improved technique
1	Regular subculturing of the strain stored in native state on beef-extract glycerol agar (every 30 days), with testing of biological properties once a year	
2	Not done	Testing of viability and biological properties (once every 5 years) of a freeze-dried strain culture
3	Passage in sensitive models (golden hamsters) once a year	Passage in sensitive models (golden hamsters) once 5 years
4	Not done	Freeze-drying of the isolated culture after passage
5	Testing of the cultural properties and colony morphology by inoculation on beef-extract glycerol agar, beef-extract glycerol broth and Pavlovsky potato agar	
6	Testing of tinctorial properties and cell morphology by microscopy of Gram-stained smears	
7	Testing of cell motility and Brownian motion by microscopy of culture in the hanging drop	
8	Indole formation	
9	Testing for catalase	
10	Formation of hydrogen sulfide	
11	Testing of fermentative activities by inoculation in Hiss' medium and skimmed milk	
12	Inactivation of the strain by autoclaving at 120 °C for 15 minutes	Inactivation of the strain by gamma irradiation at 30 kGy for 2 hours
13	Not done	Testing of agglutination properties in plate AT using glanders serum
14	Testing of pathogenicity by inoculation of culture to golden hamsters	
15	Testing of antigenic properties and antigenic specificity by immunization of rabbits and serum testing by plate AT and CFT	

culture was isolated, which was tested for authenticity and put in further storage in native state, with subsequent sub-culturing on the beef-extract glycerol agar every 30 days.

The previous strain maintenance technique of the *Burkholderia mallei* 5584 (Master seed) involved its storage in its native state, which facilitated accumulation of genetic mutations and, ultimately modification of bacterial cell properties [22]. Since the primary task of microbial collections is to preserve strains in an unchanged state for a long time, a freeze-drying stage was added. The freeze-drying step included the following:

– freezing of the culture for 12 hours;

– transfer of the culture to the freezer, plate temperature –52 °C;

– vacuuming;

– freeze-drying in automatic mode for 12 hours;

– heating (p) after 17 hours from the moment of culture loading at the following parameters: plate temperature +10 °C, ambient temperature 0 °C;

– heating (p + 1) after 18 hours with the following parameters: plate temperature +20 °C, ambient temperature +5 °C, vacuum 0.5 trr;

– end of drying after 24 hours: plate temperature +32 °C, ambient temperature +25 °C, vacuum 0.05 trr.

After drying, the bacteria were tested for viability and compliance with the data in the accession form, then put into storage in a freeze-dried state at +4 °C and after 5 years the viability and biological properties were tested [30].

In parallel, the native strain culture was maintained with sub-culturing every 28–30 days on beef-extract glycerol agar and comparative testing of biological properties was performed once a year.

After storage of the *Burkholderia mallei* 5584 (Master seed) for 5 years using an improved maintenance technique, it was found that it retains its viability, without loss or change of its morphological, biochemical, serological and virulent properties.

Thus, the improved maintenance technique for *Burkholderia mallei* 5584 (Master seed) includes the steps shown in Table 2.

The use of the freeze-drying allowed to increase the storage terms of the *Burkholderia mallei* 5584 (Master seed) and ensure the stability of its biological properties throughout the entire storage period.

In addition, freeze-drying allowed passaging of the strain in sensitive laboratory animals once every 5 years, which is more expedient and safe from an economic and biological point of view.

Handling of strains of pathogenicity groups I–II (hazard) poses a threat of potential escape of a biological agent into the production area air, human habitat and personnel infection. Reliable methods for their inactivation must be developed for this purpose. In our case, the gamma irradiation method was used. At the same time, the optimal dose of 30 kGy was determined, which made it possible to inactivate the strain and preserve the structural integrity of bacterial cells, being the antigens for hyperimmune sera preparation and serological reactions.

Thus, the improved technique for the *Burkholderia mallei* 5584 (Master seed) maintenance meets modern requirements for collections of pathogens of highly dangerous diseases for a long periods.

CONCLUSION

Thanks to the work performed, the technique for the *Burkholderia mallei* 5584 (Master seed) maintenance was improved, ensuring the preservation of its biological properties necessary for the production of high-quality laboratory diagnostic agents used during annual diagnostic tests for the timely detection of diseased animals.

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