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Long-term storage of C-141 reference strain of melioidosis agent (*Burkholderia pseudomallei*)

E. A. Artemeva[1], L. A. Melnikova[2], A. P. Rodionov[3]

Federal State Budgetary Scientific Institution "Federal Center for Toxicological, Radiation, and Biological Safety" (FSBSI "FCTRBS-ARRVI"), Kazan, Russia

[1] *https://orcid.org/0000-0002-6204-6077, e-mail: artemevaelena21@mail.ru*

[2] *https://orcid.org/0000-0002-0159-3843, e-mail: vnivi@mail.ru*

[3] *https://orcid.org/0000-0003-0853-5678, e-mail: alexandrvetspets@gmail.com*

SUMMARY

Melioidosis is a highly dangerous infectious disease caused by Hazard Group II bacteria *Burkholderia pseudomallei*, against which specific prevention and treatment tools have not been developed yet. Both humans and animals suffer from the disease. Previously the disease was prevalent in Southeast Asia regions, but currently is reported almost in all continents of the globe. Potential possibility of the agent introduction to the Russian Federation as well as the risk of malevolent use of this agent as a tool of bioterrorism dictates the need for storage of this pathogen in the microorganism collections to study its properties, develop and test diagnostic, detection and identification means. Microorganism Collection Laboratory of the FSBSI "FCTRBS-ARRVI" is responsible for storage and preservation of *Burkholderia pseudomallei* C-141 reference strain, submitted by Federal State Scientific Institution "Russian Research Anti-Plague Institute "Microbe" under the Rospotrebnadzor (Saratov city) for research purposes in 1983 and as a back-up strain in case of its loss by other collections. The purpose of the work was to study the preservation of biological properties of freeze-dried *Burkholderia pseudomallei* C-141 strain after 11 years of storage. It was established that under optimal storage conditions (temperature of 4–8 °C, skimmed milk as a cryoprotectant) the strain remained viable and retained its biological properties during the whole observation period. C-141 strain showed saccharolytic, oxidase, catalase and proteolytic activities, did not generate hydrogen sulphide, which is consistent with the melioidosis agent biochemical features. The strain was refreshed by passaging in golden hamsters and Burkholderia culture was isolated and freeze-dried. *Burkholderia pseudomallei* C-141 freeze-dried strain was tested for quality parameters, records were made and the strain was deposited.

Keywords: particularly dangerous diseases, melioidosis, strain, storage, passage, lyofilyzation

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For correspondence: Alexander. P. Rodionov, Junior Researcher, Laboratory for Collection of Strains of Microorganisms, FSBSI "FCTRBS-ARRVI", 420075, Russia, Republic of Tatarstan, Kazan, Nauchny Gorodok-2, *e-mail: alexandrvetspets@gmail.com.*

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Опыт длительного хранения референтного штамма С-141 возбудителя мелиоидоза (*Burkholderia pseudomallei*)

Е. А. Артемьева[1], Л. А. Мельникова[2], А. П. Родионов[3]

ФГБНУ «Федеральный центр токсикологической, радиационной и биологической безопасности» (ФГБНУ «ФЦТРБ-ВНИВИ»), г. Казань, Россия

[1] *https://orcid.org/0000-0002-6204-6077, e-mail: artemevaelena21@mail.ru*

[2] *https://orcid.org/0000-0002-0159-3843, e-mail: vnivi@mail.ru*

[3] *https://orcid.org/0000-0003-0853-5678, e-mail: alexandrvetspets@gmail.com*

РЕЗЮМЕ

Мелиоидоз – особо опасное инфекционное заболевание, вызываемое бактериями *Burkholderia pseudomallei*, относящимися к II группе патогенности, против которого не разработаны специфические средства профилактики и лечения. Мелиоидозом болеют как люди, так и животные. Ранее заболевание было распространено в районах Юго-Восточной Азии, в настоящее время регистрируется почти на всех континентах земного шара. Потенциальная возможность завоза возбудителя мелиоидоза на территорию Российской Федерации, а также опасность преднамеренного применения его в качестве средства биологического терроризма диктует необходимость содержания данного патогена в коллекциях микроорганизмов для проведения исследований по изучению его основных свойств, разработке и испытанию средств диагностики, индикации и идентификации. Лаборатория коллекции штаммов

микроорганизмов ФГБНУ «ФЦТРБ-ВНИВИ» осуществляет хранение и поддержание референтного штамма С-141 *Burkholderia pseudomallei*, полученного из ФКУН Российский научно-исследовательский противочумный институт «Микроб» Роспотребнадзора (г. Саратов) в 1983 г., для проведения научно-исследовательских работ, а также в качестве дублирующего штамма в случае утраты его в других коллекциях. Целью работы являлось изучение сохранности биологических свойств штамма С-141 *Burkholderia pseudomallei* после 11 лет хранения в лиофилизированном виде. Установлено, что при оптимальных условиях хранения (температура от 4 до 8 °С, криопротектор – обезжиренное молоко) штамм сохранял свою жизнеспособность и биологические свойства в течение всего срока наблюдения. Штамм С-141 обладал сахаролитической, оксидазной, каталазной и протеолитической активностью, сероводород не образовывал, что соответствует биохимическим признакам возбудителя мелиоидоза. Проведено освежение штамма путем пассажа через организм золотистых хомячков, выделена культура буркхольдерий, которая была лиофилизирована. Лиофилизированный штамм С-141 *Burkholderia pseudomallei* был проверен по показателям качества, на него оформлен паспорт, штамм заложен на хранение.

Ключевые слова: особо опасные болезни, мелиоидоз, штамм, хранение, пассаж, лиофилизация

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Для корреспонденции: Родионов Александр Павлович, младший научный сотрудник лаборатории коллекции штаммов микроорганизмов ФГБНУ «ФЦТРБ-ВНИВИ», 420075, Россия, Республика Татарстан, г. Казань, Научный городок-2, *e-mail: alexandrvetspets@gmail.com*.

INTRODUCTION

The strains of microorganisms are stored and maintained in public and national collections existing on the premises of research institutions. The collections include from hundreds to thousands or more of storage units. They are formed by the microbial strains submitted by biosecurity organizations, veterinary, medical, phytosanitary and other institutions, conducting microbiological tests [1, 2]. The main task of collections of microorganisms is to maintain the cultures in conditions that exclude their loss, change or degradation of morphological, biochemical, serological and toxic properties, as well as sensitivity to antibiotics during long-term storage [3, 4]. The collections include back-up, archival, field, reference and production strains and are the national heritage of the country, which ensure its biological and food security [5]. The microbial strains stored in the collections are needed for fundamental and applied scientific research, the development of therapeutic, diagnostic and preventive drugs, as well as for the development of modern test-kits, immunobiological products and therapeutic drugs against infectious diseases reported on the territory of the Russian Federation (anthrax, brucellosis, etc.), as well as against diseases, posing a potential risk of being introduced into the country from infected regions [6]. One of such infections is the highly dangerous disease melioidosis, which is endemic throughout Southeast Asia region [7]. In this regard, it is impossible to exclude the threat of introduction of this zoonosis into the territory of our country, as well as the risk of acts of sabotage using *Burkholderia pseudomallei* as an agent of biological terrorism. This creates the need to store strains of this microorganism in collections, to maintain and study the biological properties of the pathogen [8]. The laboratory of the microorganism strain collection of the FSBSI "FCTRBS-ARRVI" is responsible for maintaining the collection of *B. pseudomallei* strains, being a potentially dangerous biological agent.

The purpose of this study was to study the preservation of the biological properties of freeze-dried *B. pseudomallei* strain C-141 after 11 years of storage.

MATERIALS AND METHODS

Biological safety. The work was conducted in the Microorganism Collection Laboratory of the FSBSI "FCTRBS-ARRVI" in accordance with SanPiN 3.3686-21[1].

Strains. Freeze-dried *B. pseudomallei* reference strain C-141 (isolated from the patient's blood in 1948 in Saigon), received in accordance with the established procedure from the FSSI "Russian Research Anti-Plague Institute "Microbe" under the Rospotrebnadzor (Saratov city) in 1983 was used in this work.

Nutrient media. To revive the freeze-dried culture of the strain under study and to study the preservation of its morphological properties, meat peptone glycerol agar (MPGA) and meat peptone glycerol broth (MPGB) were used.

To detect the formation of hydrogen sulfide, MPGB with 4% glycerol was used; MPGA with 4% glycerol was used to study catalase activity. The above-mentioned nutrient media are produced by the FSBSI "FCTRBS-ARRVI".

Biochemical properties were analyzed using Hiss' media (NPO Microgen, Russia).

Laboratory animals. Five golden hamsters were used to revive the strain. All animal experiments were conducted in strict accordance with the interstate standards for

[1] SanPiN 3.3686-21 Sanitary and epidemiological requirements to prevent infectious diseases: approved by Resolution of the Chief Medical Officer of the Russian Federation No. 4 dated 28.01.2121. Available at: http://vnipchi.rospotrebnadzor.ru/s/203/files/ND/safety/95493_64.pdf.

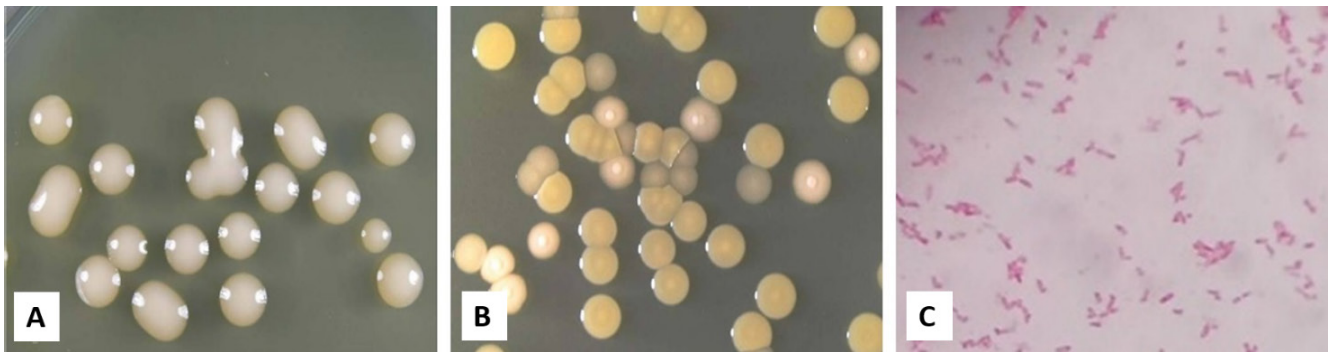

Fig. Cultural and morphological properties of B. pseudomallei C-141 strain: A – day-old culture, grown on meat peptone agar; B – dissociated culture in 48–72 hours of incubation; C – Gram stained smear of the tested culture

laboratory animal handling adopted by the Interstate Council for Standardization, Metrology and Certification, as well as in accordance with the requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS No. 123, Strasbourg, 18.03.1986).

Equipment. The culture was freeze-dried using LZ-9.2 freeze-dryer (Frigera, Czech Republic).

Test methods. After the viability of the strain was established and second generation culture was obtained by passaging, its biological properties were studied for compliance with specifications according to MU 4.2.2787-10 "Laboratory Diagnostics of Melioidosis" [9]. To study the cultural characteristics, the strain was inoculated on MPGA and MPGB. Tinctorial and morphological properties were determined by microscopy of the preparations based on a 2-day culture fixed in a Nikiforov mixture and stained according to Gram. The mobility of bacterial cells was established by microscopic examination of native preparations made by the hanging drop technique. The formation of saccharolytic enzymes was determined using Hiss' media. The proteolytic properties were tested by seeding the culture using inoculating loop in 12% gelatin, and then 2–3 drops of culture in saline solution were added to skimmed milk. The results were recorded during 3 days. The formation of hydrogen sulfide was determined in MPGB using an indicator paper soaked in lead acetate and inserted into a test tube. The oxidase activity was analyzed by applying a 1% solution of hydrogen peroxide to the surface of a culture grown on MPGA. The final reading of the results was performed on 7–10th day.

After testing the basic properties of *B. pseudomallei* C-141 strain, it was passaged in golden hamsters by subcutaneous injection of a 2-day agar culture suspension. The animals were monitored for 5–6 days. The dead animals were autopsied and samples from liver, spleen, lungs, injection site, heart blood were inoculated onto MPGA and MPGB, which were cultivated at 37 °C for 3–4 days [10].

A pure culture of the *B. pseudomallei* C-141 strain was lyophilized using skimmed milk as a cryoprotector. The freeze-drying conditions were designed for this type of pathogen.

The lyophilized culture was tested for its viability, its basic properties were studied using the data obtained, a data sheet of the strain was issued, reports were filled out, and then ampoules with the strain were deposited.

RESULTS AND DISCUSSION

Replenishment of the laboratory's collection of microbial strains of the FSBSI "FCTRBS-ARRVI" with the *B. pseudomallei* C-141 strain was dictated by the need to develop methods for differential diagnosis of glanders and melioidosis, since the causative agents of these particularly dangerous infectious diseases are antigenically closely related. In addition, this strain is deposited in the collection as a back-up strain in case it is lost in other collections. For 38 years, the strain has been stored in native state on MPGA (working cultures) and in lyophilized form and has been periodically subjected to testing for its viability and biological properties. The culture of the strain stored in its native state is re-inoculated onto MPGA every 3 months and tested for its basic properties. Lyophilized cultures are tested once every 5 years.

Currently, the storage of the *B. pseudomallei* C-141 strain in the collection and the relevant works are dictated by the ongoing globalization in all spheres of human activity, the development of tourism, especially in tropical climate areas, international relations in the field of trade, sports, etc., creating a risk of introducing exotic infections on the territory of our country, including melioidosis. Being a biological agent, *B. pseudomallei* creates a potential threat of its deliberate use as a means of biological terrorism. Melioidosis was included in the list of socially significant hazardous diseases by Decree of the Government of the Russian Federation No. 715 of December 1, 2004 concerning approval of the list of socially significant diseases and the list of hazardous diseases[2].

At the first stage, the cultural properties of the *B. pseudomallei* C-141 strain stored for 11 years in freeze-dried form, were studied. After 24 hours of cultivation at a temperature of 37 °C, small, translucent, convex colonies of grayish color with smooth edges and a smooth surface grew on the MPGA (Fig. A). After 48–72 hours of incubation, signs of culture dissociation appeared: some colonies were transparent, others had a folded surface (Fig. B). Growth manifested by light turbidity was recorded on the MPGB after a day; in the following days the turbidity increased, a precipitate and a surface film formed, which after 4–5 days became folded, changing its color from gray-yellow to brown.

[2] Concerning the approval of the list of socially significant diseases and the list of hazardous diseases: approved by Decree of the Government of the Russian Federation No. 715, dated 01.12.2004. Available at: https://docs.cntd.ru/document/901916651.

Microscopy of smears prepared from a 2-day old agar culture of the strain, fixed in the Nikiforov mixture and Gram stained demonstrated small, gram-negative cells located singly, in pairs and in short chains with characteristic bipolar staining (Fig. C). When viewed under a microscope of the "hanging drop" preparation made from *Burkholderia* culture, the rectilinear movement of bacteria was recorded.

Testing of the biochemical properties of the *B. pseudomallei* C-141 strain showed that it had a saccharolytic activity: it oxidized glucose and changed the color of the indicator and the nutrient medium. The inoculations on MPGB did not generate hydrogen sulfide: the indicator paper impregnated with lead acetic acid did not turn black. The strain had oxidase, catalase (when 1 mL of 1% hydrogen peroxide solution was applied to the *Burkholderia* culture grown on MPGA, gas bubbles appeared) and proteolytic (it coagulated and peptonized milk, liquefied 12% gelatin) activity (Table).

The results of the test showed that the biochemical properties of the C-141 strain are consistent with the species characteristics of *B. pseudomallei* provided for by the Bergey's manual [11]. This fact suggests that the strain optimal storage conditions and the work carried out for 38 years contributed to the preservation of its viability without loss of its biological properties.

To maintain the basic properties of strains during their long-term storage, it is necessary to revive them in susceptible animal models [12]. For melioidosis agent, such models are golden hamsters, white mice and guinea pigs. Golden hamsters were used in this work. The disease in these animals proceeds in an acute form, death occurs within 3–5 days, depending on the strain and the dose, besides these animals are convenient for maintenance and care. *B. pseudomallei* C-141 strain was passaged by subcutaneous injection of live culture to golden hamsters at a dose of 10^9 live microbial cells per 1 cm^3 suspended in saline solution. Then the hamsters were monitored. Depression, lethargy, anorexia, death on day 4–6 were registered in animals. During the autopsy, necrotic nodules, 2–3 mm in diameter, were found in the liver, spleen and lungs. Bacteriological testing of samples from internal organs and heart blood revealed a culture with *B. pseudomallei*-consistent biological properties.

The primary task of microbial collections is to preserve the strains in an unchanged state for a long time. For this purpose freeze-drying is most often used [13]. The isolated culture of the *B. pseudomallei* C-141 strain was lyophilized to preserve the basic properties for a long time. Skimmed milk was used as a cryoprotector, which is effectively used for freeze-drying of glander pathogen strains [14].

Lyophilization was carried out according to the previously optimized conditions:

1. Freezing of the material for 18 hours to minus 40 °C in the freezer.
2. Transfer of the material to the freeze-dryer, cooling the plate to minus 52 °C.
3. Vacuumization.
4. Lyophilization in automatic mode for 12 hours.
5. Turning on heating (p) after 17 hours from loading of the material with the following parameters: plate temperature 10 °C, medium temperature 0 °C.

Table
C-141 *B. pseudomallei* biochemical properties

No.	Parameter	of tested strain	in Bergey's manual
1	Glucose oxidation	+	+
2	Hydrogen sulfide formation	–	–
3	Oxidase activity	+	+
4	Milk coagulation	+	+
5	12% gelatin liquefaction	+	+

6. Turning on heating (p + 1) after 18 hours with the following parameters: plate temperature 20 °C, medium temperature 5 °C, vacuum 0.5 trr.
7. Next 24 hours: plate temperature 32 °C, medium temperature 25 °C, vacuum 0.05 trr.

The freeze-drying process was completed at a relative humidity of the material in the range from 2 to 3.5% [15]. Testing of the *B. pseudomallei* C-141 strain after lyophilization showed its viability and preserved basic properties.

After refreshing the strain by passaging in susceptible animal models, lyophilization and quality control in accordance with SanPiN 3.3686-21, a data sheet was issued and corresponding entries were made in the registration form No. 517/u "Card of individual registration of a collection pathogenic biological agent", after which the strain was deposited.

CONCLUSION

The results of the study showed that under optimal storage conditions (temperature from 4 to 8 °C, skimmed milk as a cryoprotector), the *B. pseudomallei* lyophilized C-141 strain retained its viability and biological properties for 11 years of storage (observation period). The strain had saccharolytic, oxidase, catalase and proteolytic activity, did not generate hydrogen sulfide, which is consistent with biochemical features of the *B. pseudomallei* species.

Refreshing of the strain by passaging in susceptible animal models (golden hamsters) and preservation by lyophilization will ensure its maintenance in a viable state and preservation of its basic properties for a long period of time.

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INFORMATION ABOUT THE AUTHORS / ИНФОРМАЦИЯ ОБ АВТОРАХ

Elena A. Artemeva, Candidate of Science (Veterinary Medicine), Head of Laboratory for Collection of Strains of Microorganisms, FSBSI "FCTRBS-ARRVI", Kazan, Russia.

Lilia A. Melnikova, Candidate of Science (Veterinary Medicine), Associate Professor, Leading Researcher, Laboratory for Collection of Strains of Microorganisms, FSBSI "FCTRBS-ARRVI", Kazan, Russia.

Alexander P. Rodionov, Junior Researcher, Laboratory for Collection of Strains of Microorganisms, FSBSI "FCTRBS-ARRVI", Kazan, Russia.

Артемьева Елена Александровна, кандидат ветеринарных наук, заведующий лабораторией коллекции штаммов микроорганизмов ФГБНУ «ФЦТРБ-ВНИВИ», г. Казань, Россия.

Мельникова Лилия Арсентьевна, кандидат ветеринарных наук, доцент, ведущий научный сотрудник лаборатории коллекции штаммов микроорганизмов ФГБНУ «ФЦТРБ-ВНИВИ», г. Казань, Россия.

Родионов Александр Павлович, младший научный сотрудник лаборатории коллекции штаммов микроорганизмов ФГБНУ «ФЦТРБ-ВНИВИ», г. Казань, Россия.