

SCIENCE AND PRACTICE



Original Study Article

<https://doi.org/10.36233/0372-9311-673>

Improvement of the bacteriological method for isolation of *Listeria monocytogenes*

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Abstract

Introduction. Currently, listeriosis is regarded as one of the dangerous infections that cannot be prevented by vaccination and is characterized by the severity of the clinical process and high mortality. Improving laboratory diagnostic methods especially in listeriosis meningitis to identify the pathogen in the shortest possible time remains an urgent problem.

The aim of the study was to investigate the behavior of collection and clinical strains of various listeria species on GBM-agar — a nutrient medium for isolating pathogens of purulent bacterial meningitis — in order to improve the bacteriological method for isolating and identifying *Listeria monocytogenes*.

Materials and methods. In the current study, 1125 samples of clinical material and food produces were used. Of these, 95 were isolated and 5 were reference strains of *Listeria* spp. The following culture media to isolate listeria were used: Agar *Listeria* by Ottaviani and Agosti (ALOA); *Listeria* enrichment broth (LEB), *Listeria* isolation agar (LIA), GBM-agar.

Results. Of the 1125 samples involved the following strains were isolated using LEB, LIA and ALOA media: *L. monocytogenes* — 89, *L. welshimeri* — 2, *L. innocua* — 3, *L. seeligeri* — 1. All isolates and reference strains were subcultured by using conventional selective media (LIA, ALOA) and additionally GBM-agar modified by adding a selective additive to isolate *L. monocytogenes*, and yolk emulsion. Colonies grown on the modified GBM-agar and belonging to the *Listeria* genus were larger and had distinctive morphological traits making them differ from colonies obtained by means of conventional listeriosis media. This allowed for the primary differentiation of *L. monocytogenes* from non-pathogenic listeria species and some other pathogens of purulent bacterial meningitis.

Conclusion. It is shown that the algorithm of the culture method can use a new nutrient medium (modified GBM agar) possessing improved growth properties for *L. monocytogenes*, the introduction of which will serve as an additional effective means for differentiating listeria during research in sanitary and clinical microbiology.

Keywords: listeria, *Listeria monocytogenes*, nutrient media, modified GBM-agar, food-borne infection

Ethics approval. The study was conducted with the informed consent of the patients or their legal representatives. The research protocol was approved by the Ethics Committee of the State Research Center for Applied Microbiology and Biotechnology (protocol No. БП-2025/3, May 17, 2025).

Funding source. The research was carried out within the framework of the industry program of Rospotrebnadzor.

Conflict of interest. The authors declare no apparent or potential conflicts of interest related to the publication of this article.

For citation: Khramov M.V., Domotenko L.V., Polosenko O.V., Mitsevich I.P. Improvement of the bacteriological method for isolation of *Listeria monocytogenes*. *Journal of microbiology, epidemiology and immunobiology*. 2025;102(3): 362–369.

DOI: <https://doi.org/10.36233/0372-9311-673>

EDN: <https://www.elibrary.ru/QONYBM>

Усовершенствование бактериологического метода при выделении *Listeria monocytogenes*

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Аннотация

Введение. Листериоз расценивается как одна из опасных вакцинонеуправляемых инфекций, характеризующаяся тяжестью клинического процесса и высокой летальностью. Актуальным направлением остаётся совершенствование методов лабораторной диагностики, особенно при листериозном менингите, для выявления возбудителя в оптимально сжатые сроки.

Цель исследования — изучить поведение музейных и клинических штаммов различных видов листерий на ГБМ-агаре — питательной среде для выделения возбудителей гнойных бактериальных менингитов — для усовершенствования бактериологического метода при выделении и идентификации *Listeria monocytogenes*.

Материалы и методы. В работе использованы 1125 образцов клинического материала и пищевых продуктов, 95 выделенных и 5 референтных штаммов *Listeria* spp., питательные среды для выделения листерий: агар *Listeria* по Оттавиани и Агости (ALOA); питательный бульон для выделения и культивирования листерий (ПБЛ), питательный агар для выделения листерий (ПАЛ), ГБМ-агар.

Результаты. Из 1125 образцов, поступивших на исследование, с использованием ПБЛ, ПАЛ и ALOA выделены штаммы: *L. monocytogenes* — 89, *L. welshimeri* — 2, *L. innocua* — 3, *L. seeligeri* — 1. Все изоляты и тест-штаммы субкультивировали на традиционные селективные среды (ПАЛ, ALOA) и дополнительно на ГБМ-агар, модифицированный внесением селективной добавки для выделения *L. monocytogenes* и желточно-эмульсии. На модифицированном ГБМ-агаре выросшие колонии, относящиеся к роду *Listeria*, были крупнее и имели отличительные морфологические признаки от колоний, полученных на классических листериозных средах, что позволило провести первичную дифференциацию *L. monocytogenes* от непатогенных видов листерий и других возбудителей гнойных бактериальных менингитов.

Заключение. Показана возможность использования в алгоритме культурального метода новой питательной среды (модифицированный ГБМ-агар), обладающей улучшенными ростовыми свойствами в отношении *L. monocytogenes*, внедрение которой послужит дополнительным эффективным средством для дифференциации листерий при проведении исследований в санитарной и клинической микробиологии.

Ключевые слова: листериоз, *Listeria monocytogenes*, питательные среды, модифицированный ГБМ-агар, пищевая инфекция

Этическое утверждение. Исследование проводилось при добровольном информированном согласии пациентов или их законных представителей. Протокол исследования одобрен Этическим комитетом Государственного научного центра прикладной микробиологии и биотехнологии (протокол № ВП-2025/3 от 17.05.2025).

Источник финансирования. Работа выполнена в рамках отраслевой программы Роспотребнадзора.

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Для цитирования: Храмов М.В., Домотенко Л.В., Полосенко О.В., Мицевич И.П. Усовершенствование бактериологического метода при выделении *Listeria monocytogenes*. Журнал микробиологии, эпидемиологии и иммунобиологии. 2025;102(3):362–369.

DOI: <https://doi.org/10.36233/0372-9311-673>

EDN: <https://www.elibrary.ru/QONYBM>

Introduction

Listeriosis is not only a medical and social but also an economic problem. Despite the fact that in recent years the incidence of listeriosis has been kept at the level of sporadic cases, listeriosis is considered as one of the dangerous vaccine-uncontrollable infections, characterized by the severity of the clinical process and high mortality (up to 20%) [1, 2].

The incidence of listeriosis is caused by contamination and active multiplication of listeria in foodstuffs, increased susceptibility to listeria in risk groups against the background of cellular immunity disorders [1, 3]. In recent years, data have been obtained on the circulation of *Listeria* in plant, soil, and water substrates, their high adaptive capabilities in a wide temperature range, humidity, and pH of the environment. There is data on the contamination of water sources with the causative agent of listeriosis near livestock enterprises [4].

In healthy people, infection is usually asymptomatic or in the form of gastroenteritis [1, 2, 5]. In the immunocompromised elderly, pregnant women and newborns, or in patients receiving immunosuppressive therapy, listeriosis may manifest as bacteremia or sepsis, central nervous system involvement, *etc.*, leading to serious or potentially fatal illnesses, including sepsis or meningitis [2, 6, 7–12].

The clinical manifestations of these forms of the disease, including listeriosis meningitis, are nonspecific, mainly fever, headache, vomiting, and disorders of consciousness, which is similar to other types of purulent meningitis [2, 3, 6, 7].

Listeriosis is caused by gram-positive, facultatively anaerobic, enteroinvasive bacteria of the genus *Listeria*. The main causative agent of the disease in humans is *L. monocytogenes*, which is capable of causing listeriosis in animals [19]. The main causative agent of listeriosis in animals is *L. ivanovii*, which in rare cases can lead to the disease in humans [20]. There are isolated cases of listeriosis caused by *L. innocua* and *L. seeligeri* [21, 22].

In the laboratory diagnosis of listeriosis and sanitary-bacteriologic investigations, the leading role is played by the bacteriologic method using nutrient media [13].

The isolation of *Listeria* from non-sterile clinical material and foodstuffs is only possible using selective nutrient media or enrichment procedures. *Listeria* enrichment broth (LEB), Fraser broth, UVM broth are used; as selective differential diagnostic media, *Listeria* isolation agar (LIA), *Listeria* agar by Ottaviani and Agosti (ALOA), Brilliance *Listeria* agar, Oxford agar, PALCAM agar, *etc.* are used as selective enrichment media.

Among the various media, LIA and PALCAM agar media do not provide species differentiation of *Listeria*. On such media, the isolation of *Listeria* is based on their ability to hydrolyze esculin to form escu-

lentine, which in the presence of iron ions forms a black complex; as a result, *Listeria* of all species form grayish colonies with a black zone around them.

Chromogenic media (ALOA and Brilliance *Listeria* agar) with special selective and chromogenic additives allow to isolate *Listeria* of different species in the form of blue-green colonies and differentiate *L. monocytogenes* and certain strains of *L. ivanovii* from other *Listeria* species by formation of a characteristic halo around the colonies due to the ability to produce phospholipase C.

The selectivity of the media with respect to associated microflora is ensured by the inclusion of lithium chloride, acriflavine, cycloheximide, nalidixic acid, polymyxin and other antibiotics [14].

For the isolation of *Listeria* from normally sterile biological substrates (blood, cerebrospinal fluid, *etc.*), which is typical in the bacteriological study of meningitis, blood and chocolate agar can be used, as well as GBM-agar, a nutrient medium for the isolation and cultivation of agents of purulent bacterial meningitis without selective additives [15]. *Listeria* on these media grows as round convex translucent non-pigmented colonies with a smooth surface after 24–48 h of cultivation.

The rich GBM-agar base containing casein hydrolysate, peptone, yeast extract, growth stimulator of hemophilic microorganisms, and glucose is able to support the growth of *Listeria* of various species.

Since the main purpose of GBM-agar is related to the cultivation and isolation of the three main pathogens of bacterial meningitis: *Haemophilus influenzae* type B, *Streptococcus pneumoniae*, *Neisseria meningitidis*, it contains selective additives only for them. Selective additives for *Listeria* have a different composition of antibiotics and contain the dye acriflavine, which has antiseptic properties and can give *L. monocytogenes* colonies a green color.

Preliminary studies of clinical material from meningitis patients have shown that the use of GBM-agar with a selective additive for *Listeria* allows isolation of the listeriosis pathogen in a shorter time than conventional *Listeria* media.

To introduce the nutrient medium into the scheme of laboratory diagnostics of listeriosis, studies using a wide range of strains belonging to *L. monocytogenes* and other *Listeria* species are necessary.

The aim of the study is to investigate the behavior of reference and clinical strains of different *Listeria* species on GBM agar to improve the bacteriological method for the isolation and identification of *L. monocytogenes* in clinical and sanitary microbiology.

Materials and methods

The following materials were used: clinical material (CSF, sectional material, blood, cerebrospinal fluid, cervical canal secretion) and food raw materials and

food products received by the testing laboratory center of the State Research Center for Applied Microbiology and Biotechnology (SRC AMB) (a total of 1125 samples); reference strains of microorganisms obtained from the State Collection of Pathogenic Microorganisms and Cell Cultures “SCPM-Obolensk”: *L. monocytogenes* 766, *L. monocytogenes* NCTC11994, *L. ivanovii* ATCC19119, *L. innocua* NCTC 11288, *L. seeligeri* ATCC 35967, *Escherichia coli* ATCC 25922, *Proteus vulgaris* HX 19 222, *Staphylococcus aureus* Wood-46, *S. pneumoniae* ATCC 6305, *N. meningitidis* ATCC 13102, *H. influenzae* ATCC 49247. Sample preparation and research were carried out using algorithms and methods recommended by SanPiN 3.3686-21¹, MG 4.2.1122-02², GOST 32031-2022³. The study was conducted with the informed consent of the patients or their legal representatives. The research protocol was approved by the Ethics Committee of the SRC AMB (protocol No. БП-2025/3, May 17, 2025).

Nutrient media produced by SRC AMB were used for *Listeria* accumulation: nutrient broth for cultivation and isolation of *Listeria* with selective additive (LEB medium) FSR 2010/09161; as differential-diagnostic — nutrient agar for cultivation and isolation of *Listeria* with selective additive (ALOA medium, SRC AMB, RU No. FSR 2010/09162); *Listeria* Ottaviani Agosti HiCynth Agar (ALOA, HiMedia, RU No. FSZ 2009/03705) with Enrichment Supplement (FD214), Selective Supplement (FD 212A); nutrient medium for isolation of purulent bacterial meningitis pathogens (GBM-agar, State Research Center AMB, RU No. RZN 2016/4872) with selective additive (polymyxin B sulfate — 0.01 g/L; nalidixic acid — 0.025 g/L; acriflavine — 0.01 g/L) (hereinafter — modified GBM-agar).

To determine lecithinase activity we used nutrient medium for quantitative determination of microbial contamination (medium No. 1 GRM, SRC AMB, RU No. FSR 2011/11415) and modified GBM-agar with ex tempore addition of 5% yolk emulsion, and the same media with the addition of activated carbon at a concentration of 0.5%.

Identification of isolates was performed on a MALDI Biotyper automatic system (Bruker Daltonik).

Results

The studies were conducted in 2 stages. The first stage was devoted to the isolation of listeriosis pathogen from clinical material, food raw materials and food products. The samples prepared for the study were seed-

ed on enrichment medium — LEB. After 24 and 48 h of incubation at $37 \pm 1^\circ\text{C}$ from LEB medium, culture fluid was seeded on special nutrient media for the isolation of *Listeria* (LIA, ALOA). After incubation, characteristic colonies, presumably belonging to *Listeria*, were selected and subcultured on GRM medium No. 1 for further identification using a MALDI Biotyper automatic system. During the study, 89 isolates of *L. monocytogenes*, 2 isolates of *L. welshimeri*, 3 isolates of *L. innocua*, and 1 isolate of *L. seeligeri* were obtained.

At the second stage, we studied the behavior of all isolates, including those from meningitis patients, and test strains of *Listeria* on GBM agar in comparison with their behavior on classical nutrient media (LIA, ALOA) under conditions of equivocation.

On LIA medium, isolated cultures and reference strains of *L. monocytogenes* formed small, grayish colonies up to 1.0 mm in diameter after 24 h; on the 2nd day their size increased to 1.2–1.4 mm. On ALOA medium, *L. monocytogenes* grew as blue-green colonies surrounded by an opaque halo 0.6–0.8 mm in diameter on the 1st day of incubation and up to 2.0 mm in diameter on the 2nd day. On modified GBM-agar already after 18 h of *L. monocytogenes* incubation, the diameter of colonies reached 1.5–2.5 mm (Fig. 1). The colonies acquired a greenish color, in contrast to the colonies obtained on the classical LIA medium.

The growth of other *Listeria* species on GBM agar differed from that of *L. monocytogenes*. When studying their growth character, museum test strains: *L. ivanovii* ATCC19119, *L. innocua* NCTC 11288, *L. seeligeri* ATCC 35967 and isolated cultures of *Listeria* species: *L. welshimeri*, *L. innocua*, *L. seeligeri* were sown on modified GBM-agar and comparison media LIA and ALOA. After 18–24 h of incubation on the modified GBM-agar, the growth of these *Listeria* species was detected in the form of smooth milk-colored colonies with a diameter of 1.6–2.0 mm, in contrast to LIA and ALOA media, on which the growth of small colonies with a diameter of 0.3–0.8 mm was observed.

When seeding from a mixture of reference strain *L. monocytogenes* 766 and *L. innocua* NCTC 11288 on modified GBM agar and subsequent incubation at $37 \pm 1^\circ\text{C}$ for 18 h, the medium was found to have differentiated properties: the growth of reference strain *L. monocytogenes* 766 was observed as greenish colored colonies with a diameter of 1.8–2.2 mm; *L. innocua* NCTC 11288 — as milky colored colonies with a diameter of 1.5–1.8 mm (Fig. 2).

Furthermore, the addition of yolk emulsion to the modified GBM-agar medium provided a clear differentiation of the reference strain *L. ivanovii* ATCC19119, which has lecithinase activity, from the reference strain *L. innocua* NCTC 11288, which does not have lecithinase (Fig. 3).

Studies of clinical material, food raw materials and food products for the presence of *Listeria* include

¹ SanPiN 3.3686-21 Sanitary and epidemiologic requirements for the prevention of infectious diseases” (enacted on 01.09.2021).

² MG 4.2.1122-02 Organization of control and methods of detection of *Listeria monocytogenes* bacteria in food products: methodological guidelines. Moscow; 2002.

³ GOST 32031-2022 Food products. Methods of detection of bacteria *Listeria monocytogenes* and other *Listeria* species (*Listeria* spp.).

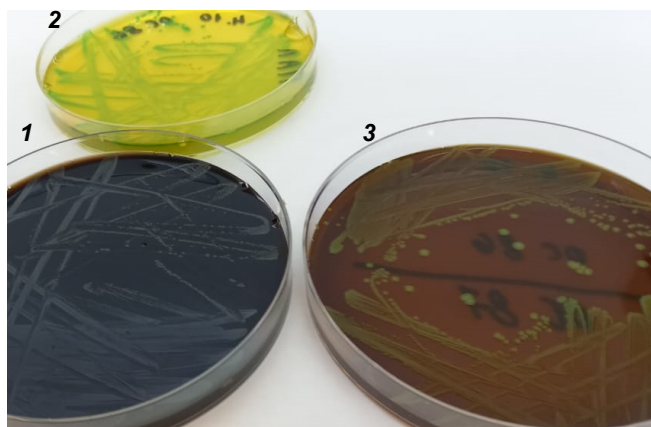


Fig. 1. Growth of isolated *L. monocytogenes* cultures on LIA (a), ALOA (b) and modified GBM agar (c).

a stage to determine the lecithinase activity, the presence of which is an indicator of pathogenicity, to confirm the affiliation of isolated bacteria to the species *L. monocytogenes*. The originality of the bacteriological method is based on the comparison of lecithinase activity of the culture in the presence or absence of activated carbon. In this study, an attempt was made to determine the lecithinase activity of *Listeria* in accordance with the method regulated by the regulatory documents MG 4.2.1122-02 and GOST 32031-2022 using modified GBM-agar with the addition of yolk emulsion with and without activated carbon. The isolated cultures of *L. monocytogenes* and reference strains *L. monocytogenes* 766 and *L. ivanovii* ATCC19119 were seeded on medium from dilutions 10^{-6} . Seeds were incubated at $37 \pm 1^\circ\text{C}$ for 24 h and viewed under transmitted light. The traditionally used for this purpose medium No. 1 GRM medium was used as a control medium of comparison.

On medium No. 1 GRM with the addition of yolk emulsion in the presence of charcoal after 24 h, a dense zone of turbidity around the colonies of isolated strains of *L. monocytogenes*, reference strain *L. monocytogenes* 766 and *L. ivanovii* ATCC19119 with a width of more than 2.0 mm, characteristic of the lecithinase activity of cultures, was observed. On the same medium without activated charcoal, no zones of opacity were observed around *L. monocytogenes* colonies, in contrast to *L. ivanovii* ATCC19119 colonies.

On modified GBM-agar with yolk emulsion without charcoal, a clearly distinguishable lecithinase activity was also observed only around *L. ivanovii* ATCC19119 colonies (Fig. 4).

Addition of activated carbon to the modified GBM-agar in the same concentrations resulted in more intense blackening of the medium, which did not allow us to consider and correctly interpret the results of lecithinase activity detection.

When determining the selective properties of modified GBM-agar, growth suppression of reference

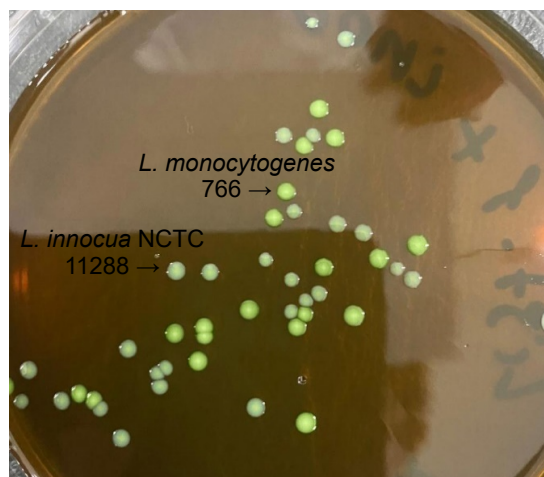


Fig. 2. Growth of a mixture of reference strains of *L. monocytogenes* 766 and *L. innocua* NCTC 11288 on modified GBM agar.

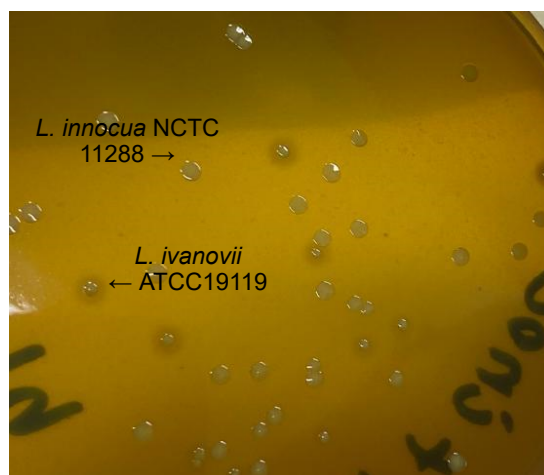


Fig. 3. Growth of a mixture of reference strains of *L. ivanovii* ATCC19119 and *L. innocua* NCTC 11288 on modified GBM agar with yolk emulsion addition.

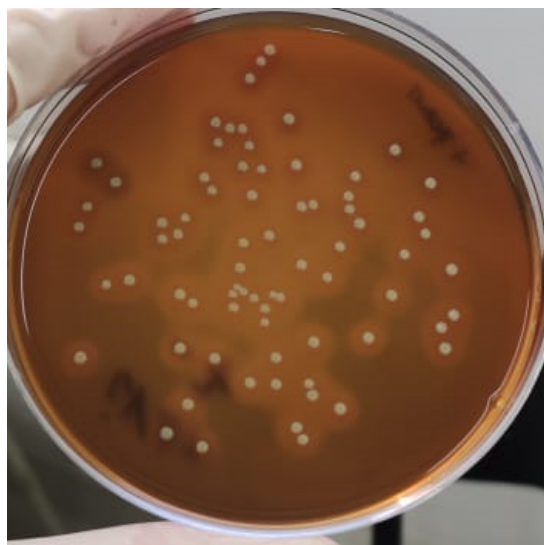


Fig. 4. Growth of reference strain *L. ivanovii* ATCC19119 on modified GBM-agar medium with yolk emulsion addition.

strains of *E. coli* ATCC 25922, *P. vulgaris* HX 19 222, *S. aureus* Wood-46, *S. pneumoniae* ATCC 6305, *N. meningitidis* ATCC 13102, *H. influenzae* ATCC 49247 from dilutions 10^{-4} was detected.

Discussion

Meningitis caused by *L. monocytogenes* is a serious and life-threatening disease. In recent years, *L. monocytogenes* is the third most common cause of bacterial meningitis in the elderly, after *S. pneumoniae* and *N. meningitidis*, because of a decrease in the incidence of meningitis caused by *H. influenzae* type B due to vaccination [18]. The disease is more common in elderly people, clinical manifestations in this type of meningitis are not specific, so for the correct diagnosis it is important to take into account the results of bacteriological examination aimed at isolation and identification of the pathogen.

The GBM-agar nutrient medium designed for isolation of the main pathogens of purulent bacterial meningitis has proven itself in the study of clinical material for the presence of *S. pneumoniae*, *N. meningitidis*, and *H. influenzae* type B [15]. Therefore, it was of interest to study the possibility of using this nutrient medium for the isolation of *Listeria*, but using a special selective additive for *Listeria*. The antibiotics polymyxin B, nalidixic acid, ceftazidime, and acriflavine included in the medium inhibit the growth of the main pathogens of GBM, as well as a number of other microorganisms (e.g., *E. coli*, *P. vulgaris*, *S. aureus*). The medium with the selective additive is called modified GBM agar.

In our study, we examined the behavior of different *Listeria* species on modified GBM agar: 89 isolates of *L. monocytogenes*, 2 isolates of *L. welshimeri*, 3 isolates of *L. innocua*, 1 isolate of *L. seeligeri*, and 5 reference strains of *Listeria* spp. On modified GBM-agar, *L. monocytogenes* colonies acquired a greenish color and after 18 h of incubation significantly exceeded the colonies on the classical LIA medium and even ALOA. At the same time, non-pathogenic *Listeria* formed small colonies of milky/cream color. This ability of modified GBM-agar will accelerate the isolation and differentiation of the main listeriosis pathogen — *L. monocytogenes* — from other *Listeria* species by morphological features of colonies (color and size).

Addition of yolk emulsion to modified GBM-agar provided clear differentiation of the test strain *L. ivanovii* ATCC19119, possessing lecithinase activity, from the reference strain *L. innocua* NCTC 11288, lacking lecithinase. However, the addition of activated charcoal, which activates the manifestation of lecithinase activity in *L. monocytogenes* on GRM medium No. 1, no zones of turbidity around *L. monocytogenes* colonies were observed. Perhaps, this situation is explained by more intensive blackening of GBM-agar, which is already colored dark brown due to the growth stimulant of hemophilic microorganisms included in its composi-

tion. And on such a dark nutrient medium it is difficult to consider and correctly interpret the results of lecithinase activity detection.

I.S. Tartakovsky and coauthors noted that identification of *L. monocytogenes* by lecithinase activity detection is often difficult due to morphological and biochemical features of the listeriosis pathogen [13]. In the studies of domestic and foreign authors it is mentioned that when cultured on nutrient media containing yolk emulsion, the lecithinase activity of *Listeria* is detected extremely weakly or is not observed at all. The addition of activated carbon can result in the sorption of the product secreted by *Listeria* that inhibits the production of lecithinase enzyme [13, 16, 17].

Conclusion

The possibility of using a new nutrient medium (modified GBM agar) with improved growth properties against *L. monocytogenes* in the algorithm of the culture method is shown. Its implementation will serve as an additional effective means for differentiating *Listeria* during research in sanitary and clinical microbiology.

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The article was submitted 21.03.2025;
accepted for publication 26.05.2025;
published 28.06.2025

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Статья поступила в редакцию 21.03.2025;
принята к публикации 26.05.2025;
опубликована 28.06.2025