MICROBIOLOGY AND VIROLOGY

COMPARISON OF THE EFFECTIVENESS OF SOLID NUTRIENT MEDIUM IN THE *IN VITRO* CULTIVATION OF *NEISSERIA GONORRHOEAE* ISOLATES

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ABSTRACT

Background. *Neisseria gonorrhoeae is a facultatively anaerobic microorganism which is extremely demanding to the composition of a nutrient media and cultivation conditions. In a situation of the increasing shortage and cost of foreign components for the preparation of solid nutrient media, it is important to study the possibility of growing hard-to-cultivate microorganisms on domestically produced nutrient media.*

The aim of the study. *To evaluate the growth of gonococcus colonies on two types of solid nutrient media – chocolate agar with growth and selective additives prepared using imported reagents and chocolate agar with growth additives manufactured by "Gem LTD" (Moscow, Russian Federation).*

Materials and methods. *A reference strain of N. gonorrhoeae NCTC 12700/ ATCC 49226 and two types of chocolate agar (the first one – prepared in the State Scientific Center of Dermatovenerology and Cosmetology using imported components and the other one – from the domestic manufacturer "Gem LTD") were used in the research.*

Results. *The equivalence of the growth properties of both studied types of nutrient media when cultivating pure gonococcus was revealed.*

Conclusions. *Ready-to-use chocolate agar with growth additives produced by "Gem LTD" can be successfully used in the laboratory for the cultivation of N. gonorrhoeae pure culture. Primary isolation of N. gonorrhoeae strains from clinical material is more appropriate to carry out on a medium that suppresses the growth of foreign microflora due to the inclusion of antibiotic additive. The organization of production of domestic bacteriological media for microorganisms with high nutrient requirements reduces the dependence of domestic microbiology on import and ensures their rapid delivery to laboratories.*

Key words: *Neisseria gonorrhoeae, nutrient medium, laboratory diagnostics, chocolate agar, selective supplement, Isovitalex, VCAT, Martin and Lewis inhibitor*

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СРАВНЕНИЕ ЭФФЕКТИВНОСТИ ИСПОЛЬЗОВАНИЯ ТВЁРДЫХ ПИТАТЕЛЬНЫХ СРЕД ПРИ КУЛЬТИВИРОВАНИИ ИЗОЛЯТОВ *NEISSERIA GONORRHOEAE* В ЛАБОРАТОРНЫХ УСЛОВИЯХ

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РЕЗЮМЕ

Обоснование. *Neisseria gonorrhoeae – факультативно анаэробный микроорганизм, крайне требовательный к составу питательной среды и условиям культивации. В ситуации нарастающего дефицита и увеличения стоимости зарубежных компонентов для приготовления твёрдых питательных сред актуальным является исследование возможности выращивать труднокультивируемые микроорганизмы на питательных средах отечественного производства.*

Цель исследования. *Проведение оценки роста колоний возбудителя гонококковой инфекции на двух типах твёрдых питательных сред – шоколадном агаре с ростовыми и селективными добавками, приготовленном с использованием импортных реагентов, и шоколадном агаре с ростовыми добавками производства российской компании ООО «Гем» (Москва).*

Материалы и методы. *В исследовании был использован контрольный штамм N. gonorrhoeae NCTC 12700/ATCC 49226 и два вида шоколадного агара: приготовленный в ФГБУ «Государственный научный центр дерматовенерологии и косметологии» Минздрава России с использованием импортных компонентов и от отечественного производителя ООО «Гем».*

Результаты. *Была выявлена равноценность ростовых свойств исследованных питательных сред при культивировании чистой культуры гонококка.*

Заключение. *Готовый к использованию шоколадный агар с ростовыми добавками производства ООО «Гем» может успешно использоваться в лаборатории для культивирования чистой культуры N. gonorrhoeae. Первичное выделение штаммов N. gonorrhoeae из клинического материала более целесообразно проводить на среде, обеспечивающей подавление роста посторонней микрофлоры за счёт включения антибиотикосодержащей добавки. Организация производства отечественных бактериологических сред для микроорганизмов с высокими питательными потребностями снижает зависимость отечественной микробиологии от импорта и обеспечивает быструю их доставку в лаборатории.*

Ключевые слова: *Neisseria gonorrhoeae, питательная среда, лабораторная диагностика, шоколадный агар, селективная добавка, Isovitalex, VCAT, ингибитор Мартина и Льюиса*

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INTRODUCTION

Neisseria gonorrhoeae is a facultatively anaerobic microorganism which is demanding to the composition of a nutrient media and cultivation conditions. The WHO Global Health Sector Strategy on Sexually Transmitted Infections (2016–2021) [1] identifies Neisseria gonorrhoeae as one of the three most important sexually transmitted infections (STIs) that require urgent and coordinated actions at the international level. The gonococcal pathogen has also been included in the WHO's list of priority pathogens to guide and promote research and development (R&D) of new antibiotics with a high priority level [2]. The relevance of Neisseria gonorrhoeae infection is determined by its ability to cause high morbidity among people of reproductive age with a pronounced negative impact on fertility and increase the risk of co-infection with other STIs. Moreover, it is important to note the lack of specific prophylaxis and the progressive resistance of *N. gonorrhoeae* to antimicrobial agents [3].

The isolation of a pure culture of gonococcus is the classical culture method, which is still the "gold standard" for the diagnosis of gonorrhea. The principal advantage of the method is the isolation of a viable culture of *Neisseria gonorrhoeae*, which is used for further molecular-biological and genetic studies, as well as for assessing the sensitivity of the microorganism to antimicrobial agents, conducted by bacteriological method [4, 5].

The culture medium for isolation of *N. gonorrhoeae* includes an agar base providing nutrients in the form of casein and peptones, phosphate buffer to maintain pH and corn starch to neutralize toxic fatty acids that may be found in the agar. Ox-blood hemoglobin provides delivery of X-factor (hemin). Isovitalex enrichment supplement is a source of vitamins, amino acids, coenzymes, glucose, iron ions and other factors that improve Neisseria growth. Selective medium differs from conventional culture medium in that it contains antimicrobial agents (e. g., vancomycin, colistin, and nystatin or other antifungal agent) that inhibit the growth of other bacteria and fungi without inhibiting the growth of gonococci. The use of selective media favors isolation of a pure culture of Neisseria since the anatomical source of the specimen also usually contains other bacterial species, although it has been shown that in rare cases some gonococcal strains may be sensitive to the concentrations of vancomycin used [6]. The medium for the isolation of *N. gonorrhoeae* should be inexpensive but characterized by specificity and sensitivity.

In 2008, in connection with the practical implementation of the international GASP (Gonococcal Antimicrobial Surveillance Program) [7] program in Russia, the State Scientific Center of Dermatovenerology and Cosmetology of the Russian Ministry of Health developed a package of "Standard Operating Procedures" (SOPs), including "Standard Operating Procedures for Species Identification of the Gonorrhea Pathogen" [8]. The paper proposes media for the initial isolation of *N. gonorrhoeae* from clinical specimens and for subsequent culturing to determine antibiotic resistance. To isolate *N. gonorrhoeae*, chocolate agar based on GC Medium Agar Base is used. According to the SOP, bovine hemoglobin and Isovitalex growth supplement are added to the GC Medium Agar Base when preparing non-selective culture medium. VCAT (Martin and Lewis inhibitor) supplement containing vancomycin, colistin, anisomycin and trimethoprim is additionally added to obtain selective agar.

Due to the introduction of economic sanctions against the Russian Federation by the United States, the European Union and a number of other countries, the purchase of products for bacteriological high-tech nutrient media is limited, so it seems relevant to search for domestic import-substituting nutrient media. One of the national companies engaged in industrial production of ready-made nutrient media with a composition suitable for cultivation of cultures of gonococcal pathogen is "Gem LTD" (Moscow).

The aim of this study was to evaluate the efficiency of *N. gonorrhoeae* colony growth on two types of chocolate agar – produced by "Gem LTD" (specialized agar for *N. gonorrhoeae* cultivation) and medium prepared from imported reagents in accordance with the SOP.

MATERIALS AND METHODS

Two types of chocolate agar were used for growing the culture of *N. gonorrhoeae* – produced by "Gem LTD" and prepared according to the instructions in the SOP for species identification of the gonorrhea pathogen developed by the State Scientific Center of Dermatovenerology and Cosmetology of the Russian Ministry of Health.

Chocolate agar produced by "Gem LTD" is a dense nutrient medium prepared in accordance with the requirements of TU 9385-003-16665457-2013 [9]. The agar contains defibrinated sheep blood, which enriches the medium with the iron-containing pigment hemin (growth factor X). Thermostable hemin is released from erythrocytes when blood is added to the chocolate agar base at about 80 °C. To improve the growth properties of the nutrient medium, thermolabile factor V (NAD, Nicotinamide adenine dinucleotide), which is involved in oxidation-reduction (redox) reactions, is additionally added to chocolate agar cooled to 45–50 °C. The ready-to-use medium is poured into 90 mm diameter Petri dishes. The dishes with the medium are hermetically packed in polyethylene bags and stored in a dry place protected from light at a temperature of 2–8 °C for 2 months.

When preparing chocolate agar at the State Scientific Center of Dermatovenerology and Cosmetol-

ogy of the Russian Ministry of Health in accordance with the SOP, components of imported origin are used: GC Medium Agar Base is produced by Indian or American companies (Pronadisa, Thermo Fisher Scientific, Becton Dickinson); lyophilized bovine hemoglobin (Hemoglobin Bovine), Isovitalex and VCAT are purchased mainly from the American supplier, Becton Dickinson. Preparation of test portions and autoclaving of agar base and hemoglobin are carried out at the laboratory center of the State Scientific Center of Dermatovenerology and Cosmetology of the Russian Ministry of Health; additives are added immediately before pouring the dishes. 90 mm diameter dishes are used, which are packed in polyethylene bags after pouring and drying and stored at 2–8 °C for up to 3 weeks.

To control the quality of media, the reference strain of *N. gonorrhoeae* NCTC 12700/ATCC 49226, recommended for antibiotic sensitivity testing [10], was used. The strain was stored in cryo-medium at –80 °C and was seeded from the storage medium into Petri dishes with medium prepared according to the SOP one day before the study.

A saline suspension equivalent to the McFarland standard of 0.5–10^8 colony-forming units per ml (CFU/ml) was prepared from an overnight culture of the reference strain, and test inoculates of 10^4, 10^3 and 10^2 CFU/ml were prepared from it. Each inoculum was seeded on 3 dishes of chocolate agar from each manufacturer in a volume of 0.1 ml, corresponding to 10^3, 10^2 and 10 CFU. For sterility control, 3 chocolate agar dishes of each manufacturer were incubated without seeding.

The growth pattern and the number of colonies grown on the agar surface were evaluated after 24 and 48 h of incubation at 37 °C and increased (3–5 %) CO_2 content. During the study, qualitative and quantitative control of the growth of *N. gonorrhoeae* on nutrient media was performed. Qualitative control is based on the evaluation of the growth pattern of the test culture. An indicator of quantitative control was the yield of microbial cells when test inoculums were sown.

RESULTS AND DISCUSSION

After 24 h of incubation, growth of colonies up to 0.5 mm in diameter, opaque, rounded in shape was observed on all three dishes with chocolate agar produced by "Gem LTD". The number of colonies grown was directly proportional to the number sown (Fig. 1), namely: an average of 750 colonies of *N. gonorrhoeae* per dish grew on dishes with 10^3 CFU/mL seeding, 90 colonies with 10^2 CFU/ml seeding, and 7 colonies with 10 CFU/ml seeding. There was no colony growth 24 h after sowing on all the dishes with medium prepared according to the SOP, but after 48 h Neisseria colonies appeared in the following numbers: on dishes with 10^3 CFU/ml sowing the number of colonies increased to an average of 950 colonies per dish; on dishes with 10^2 CFU/ml sowing – up to 80 colonies; on dishes with 10 CFU/ml sowing – up to 7 colonies. Regardless of the culture medium, all colonies were gray, with a shiny surface, opaque, convex, rounded, 1.5–2 mm in diameter. Belonging of microorganisms to the genus *Neisseria* was confirmed by oxidase test: one drop of Tetramethyl-p-phenylenediamine dihydrochloride reagent was applied to the grown colonies; after 10 s the colonies acquired blue coloring (Fig. 2).

FIG. 1.
N. gonorrhoeae colonies on chocolate agar manufactured by "Gem LTD" 24 hours after inoculation with 10 CFU per plate

No growth of foreign microflora was observed on control dishes, which were not sown, after 24 and 48 h of incubation, which confirms the fact of absence of contamination of nutrient media at all stages of preparation.

Thus, chocolate agar – both prepared according to the SOP and produced by "Gem LTD" – provides growth and manifestation of typical culture and morphological properties of gonococcus within 24–48 hours. The later appearance of *N. gonorrhoeae* colonies on the medium prepared according to the SOP is explained by the presence of the selective VCAT supplement containing antimicrobial agents (vancomycin, colistin, anisomycin and trimethoprim), which inhibit, among other things, the growth of gonococcus.

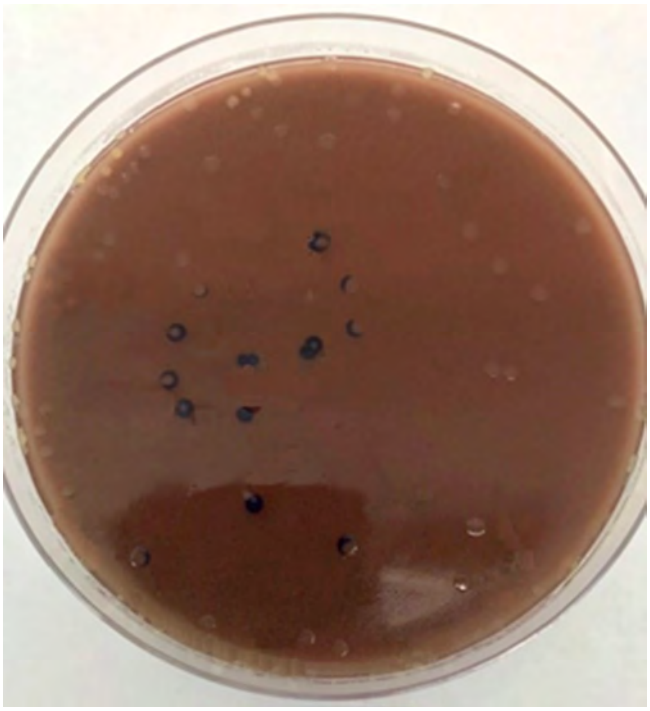

FIG. 2.
N. gonorrhoeae colonies on chocolate agar manufactured by "Gem LTD" 48 hours after inoculation with 100 CFU per plate: oxidase test

CONCLUSION

The study conducted showed the equivalence of growth properties of chocolate agar prepared according to both SOP and "Gem LTD". Both types of culture medium support the growth of the test strain of gonococcus even at as low a test inoculum level as 10^2 CFU/ml, which is important when isolating a pure culture of gonococcus from clinical material.

Ready-to-use chocolate agar with growth additives produced by "Gem LTD" can be successfully used in the laboratory for cultivation of a pure culture of *N. gonorrhoeae*, in particular for routine colony passage as part of collection or other laboratory and experimental work. When selective components are added to the agar, it can be tested for the isolation of pathogenic *Neisseria* spp. from clinical specimens as well. Thus, under the conditions of economic and other sanctions, leading to restrictions on the import of reagents for the preparation of nutrient media of foreign production, the availability of domestic media of high quality for work with difficult-to-cultivate microorganisms, which includes *N. gonorrhoeae*, is particularly important for the continuous implementation of work on monitoring antimicrobial resistance.

Conflict of interest

The authors of this article declare no conflicts of interest.

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