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Features of isolation of the anthrax pathogen depending on the type of nutrient medium

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ABSTRACT

The purpose of the work is to develop a nutrient medium for differentiation of bacillus anthracis from soil aerobic bacilli. In order to achieve the set goal, we used the method of introduction into the environment of cultivation of microorganisms separated from animals and objects of external environment (water, soil, feed, air, scrapes from different surfaces suspected of contamination by their bacillus anthracis) of nutrient substrate - sucrose, used by bacteria of Bacillus genus for synthesis of levan - product of their metabolism, a sign absent in bacillus anthracis. This feature is essential for identification and differentiation of bacillus anthracis from closely related saprophytes. To identify and differentiate the bacillus anthracis, the microbes isolated from the external environment were cultivated in a nutrient medium consisting of meat-and-peptone agar (MPA) and stimulator (inductor) synthesis of levan sucrose in the amount of 10% to 100 ml of melted agar. The proposed nutrient media was prepared as follows. Meat-peptonic agar (500 ml) was melted at 100^0C, 10 g of sucrose was added per 100 ml of medium and after the complete dissolution of sucrose, the nutrient medium was poured into Petri dishes and used for sowing the studied material for identification and differentiation of grown crops. For this purpose, soil samples taken from the territory of old cattle cemeteries were fractionally sown on MPA and thermostatted for 16-18 hours at 37^0C and examined crops for the presence of matte and rough (R-form) colonies.

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INTRODUCTION

Malignant anthrax is a dangerous infectious disease that has been known for a long time. At the same time, in spite of timely carrying out of antiepidemic and antiepizootic measures, malignant anthrax outbreaks are still observed in almost all countries of the world (Hisao and Masuo, 1989; Brabander *et al.*, 2009). Cases are registered every year not only among animals, but also among humans, including fatal ones. In laboratory diagnosis of malignant anthrax an important role is assigned to indication

and identification of the causative agent in biological material as well as environmental objects (Viñas *et al.*, 2004).

Currently, the arsenal of methods for bacillus anthracis indication and differentiation is quite wide, ranging from traditional bacteriological and animal bioassays to fluorescent antibody (FIA), indirect hemagglutination reaction (IHGA), immunoassay (ELISA) and polymerase chain reaction (PCR) (Zai *et al.*, 2013; Cristina *et al.*, 2012).

Of particular scientific interest is the development of a nutrient medium for the indication and identification of bacillus anthracis in veterinary surveillance facilities (Udalova *et al.*, 2015). The analysis of the majority of developed by now nutrient media for indication of differentiation of strains of bacillus anthracis showed, that all of them are peculiar to some or other lacks (Patent RU No. 2289622, IPC S 12 No. 1/20. Pub.20.12.2006, Bul.No. 35; Patent RU No. 2425869, IPC S 12 No. 1/20. Pub.27.05.2003, Bul.No. 10; Patent RU No. 2214453, IPC S 12 No. 1/20. Pub.10.08.2011, Bul.No. 22) (Singh, 2015). In some cases it is the instability of manifestation of differentiating features, such as phosphate formation, lecithinase production and other biochemical features of B.anthracis and closely related aerobic bacilli. In others - it is impossible to perform intraspecific differentiation of B.anthracis strains (virulent, avirulent and attenuated) (Carrasco *et al.*, 2008; Mahmoudi *et al.*, 2014; Chilumuru *et al.*, 2015).

Meanwhile, it is known that in the process of their metabolism the bacteria of the genus Bacillus, including B. sereus, B. subtilis, B. mesentericus, B. thuringiencis, B. megaterium, and B. pseudoanthracis, are able to synthesize fruittans - non-recovering polysaccharides (levan). Synthesis of levans is carried out by an enzyme - levansaccharose. When growing in a sugar-rich environment, the bacterium releases enzyme, which splits sucrose into glucose and fructose. All glucose is consumed by the microorganism, while fructose is polymerized by the enzyme to form levane.

Therefore, the synthesis of levans on the cultivation medium of the tested microorganisms can serve as one of the most important taxonomic (differential) features of closely related soil aerobic bacilli.

Lack of effective nutrient media for indication and differentiation of bacillus anthracis determines the relevance of this work.

Taking into account the proposed, the aim of the work is to develop a nutrient medium for differentiation of bacillus anthracis from soil aerobic bacilli.

MATERIALS AND METHODS

The objects of the research were nutrient media for cultivation of Bacillus genus bacteria. Meat peptone agar, Hottinger agar and meat peptone broth were used as control nutrients. The following additives were used: fermented dry peptone for bacteriological purposes (GOST 13805-76), glucose (GOST 6038-79, p.a.), sucrose (GOST 5833-75, p.a.), potassium chloride (GOST 4234-77), sodium citric acid (TU 5-09-22-48-77), crystalline violet (TU 6-09-4119-75), microbiological agar (GOST 17206-84), bromine blue (TU 6-09-2086-77); Sodium hydroxide (NaOH) (GOST 4328-77); rectified ethyl alcohol (GOST 18300-27); microbiological agar (Becton Dickinson Difco, USA); hydrogen peroxide (GOST 177-88).

The nutrient medium was prepared from dry components. The weighted amount of the dry medium were dissolved in distilled water, brought to a boil when stirring, boiled for 2-3 minutes, cooled to $(40\pm5)^0$C and poured into sterile Petri dishes or bacteriological tubes.

Microbiological studies were conducted with 3 strains of bacillus anthracis differing in virulent properties, 6 strains of soil aerobic bacilli and 3 strains of attenuated vaccines.

1) Bacillus anthracis - virulent strain 30;

2) Bacillus anthracis - virulent strain "C-7";

3) Bacillus anthracis STI-1 - vaccine strain;

4) Bacillus anthracis 55 - vaccine strain;

5) Bacillus anthracis M-71 - capsule attenuated (vaccine) strain;

6) B. cereus 8;

7) B. subtilis 433;

8) B. anthracoides 212;

9) B. megaterius 182/3;

10) B. mesenterius 66;

11) B. pseudoanthracis 104/2.

The virulence of the strains of malignant anthrax was determined by titration on white mice at subcutaneous and intraperitoneal infection.

Malignant anthrax, soil aerobic bacilli and vaccine strains were cultivated on meat-peptone broth and agar.

The obtained experimental data were statistically processed using Microsoft Office Excel 2003, "Statistica 6.0" PC software.

RESULTS AND DISCUSSION

Today, as a universal nutrient medium for both the indication and differentiation of bacillus anthracis and the etiological agent of other dangerous infections (SDI), MPA is used, the source of nitrogen in which are organic substances of meat water, products of protein separation (peptones); MPA has been known for more than 80 years and still remains the basic medium used in clinical microbiology in different countries for the purposes of sowing, cultivation and subsequent identification, provided various additives are used. In English-speaking countries the environment is known as Beef EXTRACT AGAR.

One of the promising trends in the development of rapid detection and differentiation of infectious agents is considered to be enzymatic, associated with the presence of the microorganism of certain biochemical properties that distinguish it from other representatives of this genus. This direction can be realized through the use of differential-diagnostic nutrient media, which include: a nutrient base that provides bacterial growth; a chemical substrate, the relation to which is a diagnostic attribute for a given microorganism.

In comparative assessment of the enzymatic activity of B. anthracis strains and closely related aerobic bacilli, we found a difference in their ability to synthesize fruittans - β-2.6-bound polysaccharides - levans with the enzyme - levansaccharose. This feature made it possible to carry out research to develop a dense diagnostic nutrient medium for simultaneous isolation and differentiation of anthrax cultures and spore-forming saprophytes close to bacillus anthracis.

In the course of the works the composition of the nutrient medium providing optimal conditions for growth and manifestation of biochemical activity of the microorganisms under test was selected experimentally, substrate concentrations were determined.

The proposed nutrient medium was prepared as follows. Meat-peptone agar, contained in a glass tube of 500 ml, melted in a water bath at 100^0 C, 10 grams of sucrose were added per 100 ml of medium. After complete dissolution of sugar (sucrose), the resulting nutrient medium (100 ml MPA + 10% sucrose) was poured into Petri dishes, placed in a thermostat at 37^0 C for 30 minutes for drying and release from condensation. The nutrient medium spilled into Petri dishes after drying is used for sowing the tested material for identification and differentiation of grown crops.

The nutrient medium prepared according to the method described above is sown fractionally with three dishes per culture: anthracic virulent culture B.anthracis culture, anthracic vaccine strains B.anthracis 55, attenuated capsule strain B.anthracis M-71, closely related saprophytes: B.sereus, B.subtilis, B.mesentericus, B.megaterium, B.pseudoanthracis. The crops were thermostatted at 37^0C for 16-18 hours. At the end of this time visual analysis of the grown crops was carried out.

The results of viewing the grown cultures showed that the dishes with the test medium in which malignant anthrax strains were sown showed abundant growth of typical anthrax cultures with a matte tint in all three dishes, although fractional sowing was performed.

In crops of closely related saprophytes: B.sereus, B.subtilis, B.mesentericus, B.megaterium, B.pseudoanthracis revealed a similar abundant growth of cultures throughout the whole dish and on the surface of cultures was found an abundant mucous substance in the form of droplets - levane, a product of synthesis of saprophytes close to bacillus anthracis.

The multiple repetitions of similar tests yielded identical results. Therefore, the appearance on the surface of the studied (suspicious for malignant anthrax) cultures of abundant mucous substance - levane, testifies to their belonging to the Bacillus genus, but not to the species of B.anthracis (bacillus anthracis).

Thus it is established that the kind of B.anthracis irrespective of a degree of virulence, ability to capsule formation or its absence, on the used nutrient medium does not form mucous substance - levan that is a differential criterion of an accessory of investigated cultures to B.anthracis (bacillus anthracis).

To determine the optimal ratio of the components of the nutrient medium, nutrient media with different ratios of components were prepared: MPA (80%) + sucrose (20%); MPA (85%) + sucrose (15%); MPA (95%) + sucrose (5%), MPA (90%) + sucrose (10%).

Nutrient media with the above component ratios were sown with virulent and vaccine (B.anthracis 55, STI-1) strains of bacillus anthracis, the crops were examined as described above.

It was established that with medium with minimal sucrose concentration (5%) the biomass yield was 2.1 billion in 1 ml, and with medium with both maximum (15, 20%) and optimal (10%) sucrose concentration the biomass yield was 2.5 billion / B.anthracis ml.

Consequently, an increase in sucrose concentration does not lead to an increase in biomass, so the optimal ratio of components in the proposed nutrient medium MPA - 90% and sucrose - 10%.

In the next series of experiments we tested the efficiency of the nutrient medium at indication and identification of bacillus anthracis in external environment objects.

The bacillus anthracis gets into the soil, goes through a long period of time in spore form and poses a serious problem for veterinary medicine, as the presence of the agent at the burial site of malignant anthrax corpses poses a threat of outbreak of this particularly dangerous infection. Therefore, the detection of the agent in the soil and its neutralization is the primary task of preventing this dangerous sapronosis. However, the release of the agent in the soil is associated with great difficulties due, firstly, to the insignificant concentration of the agent in the soil and, secondly, to the presence of aerobic bacilli closely related to bacillus anthracis in large quantities: B.sereus, B.subtilis, B.mesentericus, B.megaterium, B.pseudoanthracis, which by their cultural and morphological properties are similar to bacillus anthracis and which, by persisting in soil with the specified saprophytes, acquires similar properties (avirulence, acapsulogenicity, mobility, etc.). Under these conditions, existing methods and means of identification and differentiation of the agent lose effectiveness.

With this in mind, we conducted experiments on the isolation and identification of bacillus anthracis using the proposed nutrient medium.

For this purpose, soil samples taken from the territory of the cattle dumps, after treatment in accordance with the "Instruction on the separation of bacillus anthracis in the objects of veterinary inspection" were fractionally sown on MPA, thermostatted 16-18 hours at 37^0C and looked through the crops for the presence of matte and rough (R-shaped) colonies. Suspicious of malignant anthrax crops in the colony were transferred to the proposed nutrient medium, followed by thermostatting and crop viewing as described above.

It was found that out of 106 colonies selected, suspected of malignant anthrax, only 2 colonies gave abundant growth of microbes on the tested medium without the formation of abundant mucous matter - levane, while cultures from 104 colonies grown from samples of cattle-breeding soil, on the tested medium formed abundant mucous matter - levane, which indicates their belonging to the Bacillus genus and species B.B.subtilis, B.mesentericus, B.megaterium, B.pseudoanthracis which are always present in soil as well as in plants.

CONCLUSIONS

Having carried out purposeful researches on construction of differential-diagnostic nutrient medium for microbiological researches, and in particular, for indication and differentiation of bacillus anthracis from close to bacillus anthracis soil aerobic bacilli, the composition of nutrient medium providing optimal conditions for growth and manifestation of biochemical activity of microorganisms under test was experimentally selected. It has been found that the best results can be obtained by using meat-peptone agar (MPA) as the basis for the nutrient medium. The optimal concentration in the sucrose substrate medium was 10%.

In this way, the feed medium offered allows,

1. Significantly accelerate the time of bacillus anthracis excretion and identification from environmental objects;
2. Reliably differentiate bacillus anthracis from closely related aerobic saprophyte bacilli: B.sereus, B.subtilis, B.mesentericus, B.megaterium, B.pseudoanthracis species without using special nutrient media (GCI, hydrogen carbonate water, MPA with blood), expensive diagnostics (enzyme and fluorochromic antibodies), infection of susceptible animals, because these tests fully confirmed the results of identification of bacillus anthracis using the proposed sugarcane nutrient media.

The distinctive features of the proposed nutrient medium are,

1. Using meat-pepton agar as the basis for the nutrient medium of solid nutrient substrate;
2. Using sucrose as a stimulant for the synthesis of levan;
3. Exclusion of expensive Henks solution, blood serum and blood agar used in known nutrient media;
4. There is no need to study the capsule formation of the obtained crop using the described reagents and equipment, because due to the ecological interaction of bacillus anthracis with extreme factors (pH, chemical substrates, aerobic saprophytes, etc.) of soil and other objects, the capsulogenicity of the etiological agent may be temporarily or permanently absent.

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Conflict of Interest

None.

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