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Comparative Assessment of B.Anthracis Encapsulation Degree and Quality in the Nutrient SCI Depending on Crop Seed Method Applied

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Abstract.

Anthrax pathogen changes cultural, morphological, antigenic and other taxonomic characters being exposed to the influence of such environmental factors as therapeutic and preventive agents application, the microbe long persistence in a macroorganism; the effects of physical, chemical and biological factors on a pathogen. They include such main symptoms as toxigenicity and capsule formation which makes it difficult to identify the pathogen? Studies have been conducted to assess the anthrax pathogen encapsulation degree depending on the crop seed. 5 anthrax cultures-isolates were used in the experiment. They were isolated both from the body of an animal that had fallen from anthrax, experimentally infected with the pathogen and treated, as well as isolated from the unfavorable for anthrax infection soil disinfected with chlorine lime. The isolates were studied for their ability to encapsulate in vitro by source material primary seeding on a liquid nutrient medium (MPB) that were further subcultured on a special SCI medium (State Control Institute) in accordance with the existing Instruction (1980). The source material was sown on the solid nutrient medium (MPA) followed by seeding on the SCI medium for a comparative assessment of the indication method effectiveness. It also helps to identify the anthrax causative agent by means of the capsule formation test. It was stated that familiar identification method provides capsule formation in 51.1 - 81.3% of B.anthracis cells depending on the strain. The solid nutrient medium (MPA) application for the studied samples initial seeding had a stimulating effect on the encapsulation degree. The number of capsular cells increased by 1.19-1.55 times depending on the strains of the pathogen studied.

Key words: anthrax pathogen, capsule, nutrient medium.

INTRODUCTION.

Anthrax remains one of the most dangerous infectious diseases at present. Despite the progress achieved in anthrax problem studying, this infection is still quite widespread in many countries of the world. It is not uncommon in Russia and the CIS countries.

Not much attention was given to the variability of the anthrax microbe in the literature which makes it difficult to indicate and to identify the pathogen. At the same time capsule formation [1] which along with its ability to synthesize three-component anthrax toxin [2] determines pathogen virulence [3] is the most important differential sign of the causative agent which distinguishes it from aerobic saprophytes similar to it.

The pathogen forms a capsule in the body [4]. The capsule prevents phagocytosis of B. anthracis and promotes the fixation of capsule bacilli to the cells of the macroorganism [5]. Anthrax noncapsular variants lack this quality completely.

Anthrax further studies showed that bacilli also form a capsule under certain cultivation conditions on nutrient media with a high content of native protein. The capsule is formed both in liquid and dense serum-supplemented media [6].

Although a number of methods and nutrient media were proposed to isolate the causative agent in vitro, this issue has not yet found a positive solution. Thus, it is recommended to use a daily broth culture for planting on the SCI medium according to the "Instructions for Laboratory Diagnosis of Anthrax (1990)" [7] to determine encapsulation in vitro. However, ambiguous and unstable results were obtained: encapsulation had different degrees of severity depending on the degree of the studied strains virulence isolated from various objects (patmaterial, soil, water, feed samples). Optimal conditions to determin encapsulation are the following: pre-cultivation, seeding the studied material on MPA with 0.7% sodium bicarbonate or MPA with blood serum of cattle with grown culture further sowing in the liquid SCI [8].

Research aimed at optimizing the conditions for growing anthrax bacilli on the SCI medium was carried out taking into account the ambiguity of the results to determine the encapsulation degree for the studied cultures of the anthrax pathogen.

MATERIAL AND METHODS.

Meat-peptone broth (MPB), meat-peptone agar (MPA), and SCI medium were used as tested nutrient media. 1% peptone and 0.5% NaCl were added to 1 liter of meat water and boiled for 10 minutes, made alkaline with 1% sodium bicarbonate solution (Na_2HCO_3) until slightly alkaline (pH 7.4-7.0), boiled it 3-5 minutes and filtered through paper in order to prepare MPB.

In order to prepare MPA 20-30 grams of finely chopped imported agar were taken for 1 liter of MPB, the mixture was kept in an autoclave for 20 minutes at 1 atmosphere. Then the pH was adjusted and filtered hot through cotton wool.

These nutrient media were used for the initial seeding of the source material. 10 isolates from the animals' bodies as well as from soil samples taken at sites unfavorable for anthrax (Yakut ASSR, Astrakhan Region) were used as anthrax cultures test subjects. The studied isolates were sown on the test nutrient medium (MPA, MPB), the cultures were incubated for 24-42 hours at 37 °C. After this exposure, the grown cultures were transferred to the SCI medium and through various exposures (2,4,6,8,10,12,16,18,20,24 hours) smears were made from the grown cultures, stained with Leffler blue dye and viewed under a light microscope using the immersion system. The degree and nature of capsule formation was assessed by the percentage of capsogenic and titer (concentration) of the capsule-forming cells. The cell titer was detected by the limiting dilution method.

Cultivation of the anthrax microbe was carried out in flasks with a capacity of 0.5 l, filling factor (n) = 0.5, at 36–380 ° C, inoculum doses 5×10^5 spores / ml, and incubation exposure 10–18 hours.

Table –B.Anthracis Encapsulation Degree Depending on their Origin and Crop Seed Method Applied

# isolate	The source and conditions of microbe Presence in the ecosystem	The results of the microbes cultivation on the SCI medium after sowing from media			
		MPB		MPA	
		Encapsulation degree, %	Microbe titre, $X \cdot 10^x$	Encapsulation degree, %	Microbe titre, $X \cdot 10^x$
1	Excreted from persistent animal	51.1±1.7	$4.8 \cdot 10^6$	$79.72.1^{xx}$	$6.1 \cdot 10^{8\,xx}$
2	Excreted from cow's anthrax corp	78.5±2.5	$7.2 \cdot 10^7$	89.3 ± 3.3^x	$7.3 \cdot 10^{8\,x}$
3	Excreted experimentally from infected guinea pig	81.3±1.9	$7.9 \cdot 10^7$	96.7 ± 2.5^{xx}	$6.7 \cdot 10^{9\,xx}$
4	Excreted from the body of an experimentally infected guinea pig, treated with globulin and antibiotic	69.9±3.1	$6.1 \cdot 10^8$	91.9 ± 1.5^{xx}	$5.9 \cdot 10^{9\,x}$
5	Excreted from the soil treated with bleach during the anthrax elimination	71.3±2.7	$7.3 \cdot 10^7$	89.7 ± 2.1^x	$6.3 \cdot 10^{8\,x}$

x – P< 0.5; xx – P< 0.01

RESULTS AND DISCUSSION.

A comparative analysis of the results of microbiological studies on the encapsulation degree of anthrax isolates different in origin showed the following. Firstly, this indicator depends on the conditions of anthrax pathogen presence in animals and environmental factors that influence on the microorganism. Secondly, it depends on the method of bacillus cultivation using various substrates and crop seeds.

The summarized results for determining the encapsulation degree by different isolates depending on the crop seed are presented in the table.

The materials presented in the table show that the encapsulation degree of anthrax cultures and the quality of the capsule-forming cells on the SCI medium depended on the conditions of the microbe in the ecological system "microbe + medium" and the crop seed application (the medium of primary culture).

It was stated that, regardless of the studied cultures origin the use of the solid nutrient medium for the primary seeding of the test strain had a stimulating effect on the degree of capsule formation and the titer of growing cells on the SCI medium.

Thus, the degree of capsule formation was 51.1 ± 1.7% with a titer of $4.8 \cdot 10^6$mk / ml when seeding on the SCI medium after the initial growing of the culture on a liquid medium (MPB) in isolate No. 1. It was isolated from the body of a cow that suffered from the persistent form of anthrax (asymptomatic long-term persistence of the pathogen in the body – the microbe presence).

Seeding the same culture from solid nutrient medium (MPA) on the SCI medium had a stimulating effect on the encapsulation degree for the tested culture in which the studied parameter 1.56 times (P <0.01) exceeded that of seeding from a liquid medium with capsular cell titer $6.1 \cdot 10^8$ mk / ml.

Isolate No. 4 isolated from the body of a guinea pig treated with anti-siberian globulin and antibiotics had a relatively low encapsulation degree after animal's experimental infection with the anthrax pathogen.

The results of microbiological studies using liquid (MPB) and solid (MPA) nutrient media for reseeding cultures on the SCI medium showed that when the studied culture was subcultured from the MPB on SCI medium the encapsulation degree of bacilli in isolate No. 4 was 69.9 ± 3.1%. It was 91.9 ± 1.5% (P <0.01) when reseeding from MPA to the SCI medium. It is 1.31 times higher than that of the MPB with capsule-shaping cell titer $5.9 \cdot 10^9$ mk / ml against $6.1 \cdot 10^9$ mk / ml.

Isolate No. 5, isolated from the soil treated with bleach during the anthrax elimination had a weak encapsulation degree on the SCI medium after reseeding from a liquid nutrient medium (MPB).

It was stated that the encapsulation degree in isolate No. 5 when reseeding from a liquid medium (MPB) was 71.3 ± 2.7% with a microbial titer of $7.3 \cdot 10^7$ mk / ml. After reseeding from a solid medium (MPA) on SCI medium this indicator 1.26 times (P <0.05) exceeded the first variant with a microbial titer of $6.3 \cdot 10^8$ mk / ml versus $7.3 \cdot 10^7$ mk / ml when reseeding microbes from the MPB on SCI medium.

The highest encapsulation was shown by microbes from isolate No. 3, isolated from the body of a guinea pig experimentally infected with an anthrax pathogen virulent strain. The degree of microbial cell encapsulation was 81.3 ± 1.9% with a cell titer of $7.9 \cdot 10^7$ mk / ml upon reseeding this culture from MPB to the SCI medium.

The use of solid nutrient medium (MPA) as the primary substrate for growing the original culture and its further subculture on the SCI medium led to a significant increase in the encapsulation degree which was 96.7 ± 2.5% (P <0.01) with a cell titer of $6.7 \cdot 10^9$ mk/ml against $7.9 \cdot 10^7$ mk/ml from the original grown in the liquid medium (MPB).

The experimental data obtained indicate that the use of generally accepted methods for the anthrax causative agent indication and identification according to such main criterion as the capsule formation test requires new approaches.

The pathogen ecology should be taken into account. This issue is particularly acute in the context of the genetic possibilities to change microorganism's properties in natural conditions and in the pathogen presence in the body of susceptible animals as well as under the influence of therapeutic and preventive means on the carrier's body.

As can be seen from the data in the table the long-term pathogen presence in the body of a susceptible animal (cow) who had been ill without showing clinical signs of the disease (an asymptomatic persistent form of anthrax infection) had a bacterium-altering effect. It was accompanied by the capsularity inhibition which is the main species characteristic of the pathogen. It was 51.1 ± 1.7%. This fact indicates encapsulation, i.e. the splitting of the species trait when 48.9% of the pathogen cells turned out to be acapsulated. However, it was stated that the degree of B.anthracis capsule-forming cells was 79.7% when transferring the starting material from solid nutrient medium (MPA) to a special SCI medium. It 1.56 times (P <0.01) exceeded the first variant of the grown cells.

Thus, the encapsulation inhibition of the anthrax culture during the pathogen persistence in a macroorganism induced acapsulation. It is not true as 28.8% of the cells that did not form a capsule when they were transferred from the MPB to the SCI medium. Having been transferred from the MPA to the indicated medium they turned to be encapsulated.

The results obtained can be interpreted in two aspects. Firstly, the heterogeneity of the test cultures by means of encapsulation may indicate the inadequacy of the applied methods and means of pathogen indication and identification by the capsule formation test. Two nutrient media of different biochemical composition (MPB and MPA) for preliminary rearing of the test cultures show significantly different capsule formation results.

Secondly, the anthrax pathogen has a special plasticity and can change its biomorphological properties under the influence of various environmental factors. It is not always possible to state an inducing agent. Therefore in such cases we deal with a pathogen spontaneous variability. Such variants in B.anthracis can spontaneously arise in case of being cultivated on various nutrient media. These variants are capsules-free. They depend on the genetic orientation of the inducing agent. Intra- and interspecific genetic transformation can be observed by means of chromosomal and plasmid DNA as well as by transfection with phage DNA [9].

A capsular version of the avirulent capsule-free vaccine strain B.anthracis STI-1 was obtained when cultivating an acapsulated vaccine strain STI-1 on a medium containing precipitating serum and chicken eggs or edematous liquid (aggressin) [10].

Capsule substance production in virulent and some avirulent B.anthracis cells is mediated by the pXO_2 plasmid [10]. Experiments on the plasmid transfer carried out by SR-51 transduction mediated by the phage with a frequency of 10^{-8} by means of a mating system. Participation of the pXO_2 plasmid in the synthesis of the capsular substance proved it. Having acquired the plasmid pXO_2, cells of the encapsulated B. anthracis strains produced a capsule. 1% of the cells showed a loss of the pXO_2 plasmid when growing such strains in novobiocin presence (an elimination plasmid agent).

Cultures heterogeneity according to the encapsulation degree observed in our experiment is possible when two nutrient media different in biochemical composition are applied. It may be due to two probable possibilities or insufficient nutrient media used for the initial sowing (growing) of the starting material (MPB) [11]. Applied media may contain either inhibitors (elimination plasmid pXO_2 agents) or tested cultures capsulation stimulants (transdermal by the plasmid pXO_2 transfer with phages) [12].

Taking into account the above mentioned, further studies on the optimization of nutrient media that ensure maximum biosynthesis of the polypeptide capsular substance by means of microorganism's metabolism products [13] and bicarbonate magnesium-calcium mineral water [14] have been carried out.

CONCLUSION.

1. The degree of B.anthracis cells encapsulation in vitro depends not only on the genetically determined capacity for capsulation of this species but also on the cultivation conditions and composition of the nutrient medium.

2. It has been stated that liquid nutrient medium (MPB) for the primary seeding of the material under study with the grown crops further transfer on a special nutrient for capsule formation (SCI) does not provide a complete synthesis of the capsular polypeptide. The degree of capsule formation for cultures of different origin varies from 51.1 ± 1.7 to 81.3 ± 1.9%.

3. Solid nutrient medium (MPA) for the primary seeding of the studied samples has a stimulating effect on the biosynthesis of the capsular polypeptide. The cells encapsulation degree of the same cultures on the SCI medium after transfer from MPA increases to 79.7 – 96.7%. It is 1.19 – 1.55 times more than when replanting the culture grown on the MPB than on SCI medium.

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