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# Influence of bovine blood serum on growth properties of nutrient media for *Mycoplasma gallisepticum* and *Mycoplasma synoviae* cultivation

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**SUMMARY**

The growth properties of the nutrient medium for the cultivation of pathogenic mycoplasmas depend on the type of blood serum it is supplemented with. Comparative tests of two cell-free nutrient media supplemented with bovine and porcine blood sera for the cultivation of strains "S6" *Mycoplasma gallisepticum* and "WVU 1853" *Mycoplasma synoviae* were performed. Growth properties of the tested nutrient media were assessed by determining the activity of the resulting biomass in the hemagglutination and agglutination assays, as well as by determining the concentration of viable cells after the 9th passage. It has been shown that a cell-free nutrient medium supplemented with the porcine blood serum is optimal for the cultivation of pathogenic mycoplasma species causing infectious diseases in birds. The hemagglutinating activity of the *Mycoplasma gallisepticum* culture reached 5 HAU $\log_2$ after 72 hours of cultivation, the agglutinating activity of *Mycoplasma synoviae* reached 5 AU $\log_2$ during the 88-hour incubation period, the concentration of viable cells of both strains was $10^6$ CFU/cm$^3$. The low growth properties of the medium prepared with the addition of bovine blood serum are most likely associated with its biochemical composition, which contains 5–20 times more provitamin A than the porcine blood serum, and high density lipoprotein cholesterol. On the contrary, in the porcine blood serum, most of the lipoproteins have a low density, containing a large amount of fatty acids and cholesterol, which are the main structural elements of mycoplasma cells. The obtained test results are of practical value and can be used in the technology of cultivation of pathogenic species of avian mycoplasmas in the production of diagnostic and preventive tools.

**Keywords:** *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, nutrient medium, cultivation, blood serum

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# Влияние сыворотки крови крупного рогатого скота на ростовые свойства питательной среды для культивирования *Mycoplasma gallisepticum* и *Mycoplasma synoviae*

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**РЕЗЮМЕ**

Ростовые свойства питательных сред для культивирования патогенных микоплазм зависят от вида сыворотки крови в их составе. Проведены сравнительные испытания двух бесклеточных питательных сред с добавлением сывороток крови свиней и крупного рогатого скота для культивирования штаммов «S6» *Mycoplasma gallisepticum* и «WVU 1853» *Mycoplasma synoviae*. Изучение ростовых свойств испытуемых питательных сред проводили путем определения активности полученной биомассы в реакции гемагглютинации и реакции агглютинации, а также оценки концентрации жизнеспособных клеток после 9-го пассажа культивирования. Показано, что бесклеточная питательная среда с сывороткой крови свиней является оптимальной для культивирования патогенных видов микоплазм, вызывающих инфекционные заболевания у птиц. Гемагглютинирующая активность культуры *Mycoplasma gallisepticum* достигала 5 ГАЕ $\log_2$ после 72 ч культивирования, агглютинирующая активность *Mycoplasma synoviae* – 5 АЕ $\log_2$ за 88-часовой период инкубации, концентрация жизнеспособных клеток обоих штаммов была на уровне $10^6$ КОЕ/см$^3$. Низкие ростовые свойства среды, приготовленной с добавлением сыворотки крови крупного рогатого скота, вероятнее всего, связаны с ее биохимическим составом, которая содержит в 5–20 раз больше провитамина А, нежели сыворотка крови свиней, а холестерин в основном представлен липопротеинами высокой плотности. Напротив, в сыворотке крови свиней большая часть липопротеинов имеет низкую плотность, содержащих большое количество жирных кислот и холестерина, которые и являются основными структурными элементами клеток микоплазм. Полученные результаты исследований имеют практическую ценность и могут быть использованы в технологии культивирования патогенных видов микоплазм птиц при производстве средств диагностики и профилактики.

**Ключевые слова:** *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, питательная среда, культивирование, сыворотка крови

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## INTRODUCTION

Mycoplasmas are bacteria, the key difference of which from other representatives of prokaryotes is the absence of a cell wall, which explains their resistance to a number of antibacterial drugs. The variety of forms of these microorganisms includes spherical, disk-shaped, rod-shaped and filamentous structures, varying in size from 0.1 to 1.5 microns. Another feature of mycoplasmas is that during growth on nutrient media and cell division, their size is less than the theoretical limit of self-reproduction. Mycoplasmas reproduce by budding, binary fission, fragmentation, divide by cells of unequal size, as a result of which one of the newly formed cells may not be viable. Many mycoplasma species are difficult-to-cultivate microorganisms and are poorly adapted to nutrient media, which creates certain difficulties in the technology for the production of diagnostic systems and vaccine preparations [1, 2].

In the process of growth, they need amino acids (arginine, isoleucine, methionine, phenylalanine, asparagine). A need for bile salts and fatty acids has been noted. One of the media that can ensure the growth of many types of mollicutes is modified Frey's medium, which includes PPLO-broth, dextrose, pig blood serum, *β*-nicotinamide adenine dinucleotide and L-cysteine hydrochloride. This medium is a source of amino acids, carbohydrates, and various vitamins necessary for the growth of mycoplasmas [2, 3]. The blood serum for the nutrient medium is obtained mainly from pigs or horses, since the blood serum of other animals can not only slow down growth, but rather completely inhibit it. Blood serum, as a growth stimulator of mycoplasmas, is an important component of the medium, without which cultivation on cell-free nutrient media becomes impossible.

The study of the possibility of using blood sera from different animal species as part of nutrient media for mycoplasmas can be a positive experience in the issue of their laboratory or industrial cultivation.

When it comes to oxygen requirements, mycoplasmas belong either to strict aerobes or to obligate anaerobes. Their growth lasts from 3 to 7 days at first inoculations, then in the process of reinoculation they can grow significantly faster.

The cultivation of these microorganisms is an important link in the creation of diagnostic tools, specific prophylaxis, which is an alternative to antibiotic therapy in the eradication of infections of mycoplasma etiology on industrial poultry farms [4].

The problem of mycoplasmoses arose as a result of the introduction of intensive poultry rearing methods at establishments, which in turn led to their wide spread among poultry [5, 6]. Infections such as respiratory mycoplasmosis and infectious synovitis affect chickens, turkeys, pigeons, quails and partridges. Clinical manifestations of respiratory mycoplasmosis (causative agent – *Mycoplasma gallisepticum*) are respiratory system disorders (rhinitis, laryngitis, aerosacculitis, pneumonia), conjunctivitis, sinusitis, bursitis, anemia, tendovaginitis are distinguished among systemic disorders [7–9].

The economic damage caused by these diseases consists of a decrease in the laying productivity of poultry, slow growth, and a decrease in the egg hatchability. Moreover, an infection caused by *Mycoplasma synoviae* is characterized by a decrease in the quality of the eggshell – a manifestation of the vitreous apex syndrome [10]. The immunosuppressive properties of mycoplasmas make ineffective measures for the specific prevention of other economically significant diseases, preventing the development of an immune response during live vaccine administration [11–13].

The study of the influence of essential components in the medium affecting mycoplasma growth, in this case in the bovine blood serum, is a serious challenge in mycoplasmology when creating means for diagnosing and preventing the infections in question.

## MATERIALS AND METHODS

The "WVU 1853" *Mycoplasma synoviae* and "S6" *Mycoplasma gallisepticum* strains from the FGBI "VGNKI" (Moscow) were used in the study.

*Mycoplasma gallisepticum* and *Mycoplasma synoviae* were cultivated on a liquid and dense modified Frey's nutrient medium, which included distilled water, a medium for the cultivation of pleuropneumonia-like organisms (PPLO broth base), yeast extract, glucose, thallium acetate, and bovine as well as porcine blood serum [14]. Previous studies have shown that a 12% concentration of porcine blood serum in the medium ensures optimal growth of *Mycoplasma gallisepticum* and *Mycoplasma synoviae*, so the concentration of this component was not changed during the experiment. When growing *Mycoplasma synoviae*, *β*-nicotinamide adenine dinucleotide (1% solution) and L-cysteine hydrochloride (1% solution) were also added to the nutrient medium as a V-growth factor. Phenol red served as an indicator of biomass growth control. Since mycoplasmas are sensitive to the acidity of the medium, the acid-base balance during the preparation of the medium was adjusted to pH 7.8–8.0. To prevent the growth of foreign microorganisms, antibiotics (benzylpenicillin sodium salt) and thallium acetate (10% solution) were added to the medium [15].

The cultivation of mycoplasmas was carried out in a thermostat at a temperature of (37.5 ± 0.5) °C. The cultivation time depends on the type of mycoplasma. Thus, the optimal duration of *Mycoplasma gallisepticum* cultivation ranged from 48 to 96 hours, and *Mycoplasma synoviae* – from 3 to 7 days. The growth of biomass was monitored by the change in the color of the indicator and the transparency of the nutrient medium. During growth, the medium changed color from red-brown to yellow-brown and became slightly cloudy or opalescent. The growth of *Mycoplasma synoviae* was accompanied by the formation of an oil film on the surface of the medium.

The concentration of living cells of mycoplasmas was determined by titration on dense nutrient media in Petri dishes. For this, 10-fold dilutions were prepared for the culture of *Mycoplasma gallisepticum* and 5-fold dilutions for the culture of *Mycoplasma synoviae*, which were incubated for 5 days at a temperature of (37.5 ± 0.5) °C. Then the dishes were examined under a Micros MC 50 X microscope (Austria) at 200× magnification to detect characteristic colonies that looked like "fried eggs". The arithmetic mean of the colonies in several non-overlapping fields of view corresponds to the number of colony-forming units (CFU).

The calculation of CFU in the field of view of the microscope was carried out according to the formula:

$$T = N \times 10^{d} \times K / V,$$

where $T$ – CFU titre in 1 cm$^3$;

$N$ – arithmetic mean number of colonies in one field of view of the microscope;

$d$ – culture dilution rate;

$K$ – a coefficient equal to the ratio of the dish area to the area of the field of view of the microscope (the area of the field of view of the microscope was determined using a measuring glass);

$V$ – volume of culture biomass added.

When evaluating the growth properties of a cell-free nutrient medium, attention was also paid to the determination of the hemagglutinating activity of *Mycoplasma gallisepticum* and the agglutinating activity of *Mycoplasma synoviae* in the hemagglutination and agglutination reactions, respectively to solid nutrient medium [16, 17].

## RESULTS AND DISCUSSION

The main criterion in the selection of blood serum for the preparation of a nutrient medium is to obtain the

**Table 1**
**Growth properties of the cell-free nutrient medium supplemented with the bovine blood serum for *Mycoplasma gallisepticum* cultivation**

| Cell-free nutrient medium samples | Cattle blood serum contents (%) | Biomass growth rate (h), passage 1–3 | Biomass growth rate (h), passage 1–3 after cloning | Biomass growth rate (h), passage 1–3 after recloning | Culture activity at passage 9 in HA test (HAU $\log_2$) | Growth properties at passage 9 (CFU/cm$^3$) |
|---|---|---|---|---|---|---|
| 1 | 12 | 118–120 | 115–120 | 94–96 | 2.0 | $10^{3.5}$ |
| 2 | 15 | 118–120 | 115–120 | 94–96 | 2.5 | $10^{6}$ |
| 3 | 20 | 117–120 | 114–120 | 92–94 | 3.0 | $10^{5}$ |
| 4 | 25 | 115–120 | 112–120 | 90–94 | 3.0 | $10^{5}$ |
| Control (cell-free nutrient medium with pig serum) | 12 | 72–78 | 68–72 | 68–72 | 5.0 | $10^{6}$ |

maximum amount of biomass of cultivated cultures with the highest possible hemagglutinating (for *Mycoplasma gallisepticum*) and agglutinating (for *Mycoplasma synoviae*) activity.

During the experiment, 9 passages and 2 cloning procedures of both cultures were carried out at the 3rd and 6th passages. The cultures after the last passage were examined in hemagglutination and agglutination reactions to establish activity and growth properties after adaptation to a nutrient medium; at the same time passages in the control cell-free nutrient medium were performed (with porcine blood serum) to compare the results.

The criterion for selecting the type of blood serum for the preparation of the nutrient medium was based on the calculation of CFU for *Mycoplasma gallisepticum* and *Mycoplasma synoviae* under microscope on solid nutrient medium. The results of the experiment are presented in Table 1 and 2.

It was established that the cell-free nutrient medium supplemented with the bovine blood serum at a concentration of 12 to 25% ensured the growth and accumulation of *Mycoplasma gallisepticum* over 3 passages in the range from 115 to 120 hours. At the same time, the concentration of bovine blood serum in the medium did not significantly affect its growth properties. When using porcine blood serum at a concentration of 12% in the cell-free nutrient medium the cultivation time was reduced by 40–45 hours already at passage 1, which is an important factor in the technology for the production of diagnostics and prevention of infectious diseases of birds of mycoplasmal etiology.

To obtain an axenic culture of the microorganism at passage 3, cloning was performed on Frey's dense nutrient medium with thallium acetate. The use of the *Mycoplasma gallisepticum* clone enhances the adaptation of the microorganism to such new conditions as changing the composition of the culture medium, which in turn reduces the cultivation time and increases the concentration of mycoplasma cells in the biomass volume. Thus, after repeated cloning at the passage 3, the growth time significantly decreased from 120 to 96 hours.

When evaluating the hemagglutinating properties of *Mycoplasma gallisepticum* culture in HA test, it was found that after the 9th passage, the activity of the culture grown on the medium supplemented with bovine blood serum ranged from 2.0 to 3.0 $\log_2$, which is a relatively low indicator compared to the growth of the culture on the control medium with the porcine blood serum, where the HAU was 5.0 $\log_2$.

The calculation of living cells in 1 $cm^3$ of the nutrient medium showed that their lowest concentration ($10^{3.5}$ CFU/$cm^3$) was observed when using a medium containing 12% of bovine blood serum, while when using porcine blood serum (12%), the concentration of mycoplasma cells was the highest and amounted to $10^6$ CFU/$cm^3$. An increase in the concentration of cattle blood serum in the medium from 15 to 25% had a positive effect on its growth properties, while the CFU titer was in the range of $10^5$–$10^6$ per 1 $cm^3$. However, the quality of the resulting antigen is determined not only by the concentration of cells in the volume of the medium, but by a set of indicators, including growth time, hemagglutinating and agglutinating activity, CFU. Thus, the porcine blood serum in Frey's nutrient medium ensures the maximum accumulation of the *Mycoplasma gallisepticum* culture with a minimum cultivation time.

Figure 1 shows micrographs of *Mycoplasma gallisepticum* colonies grown on a nutrient medium with bovine blood serum. The colonies of the pathogen in the field

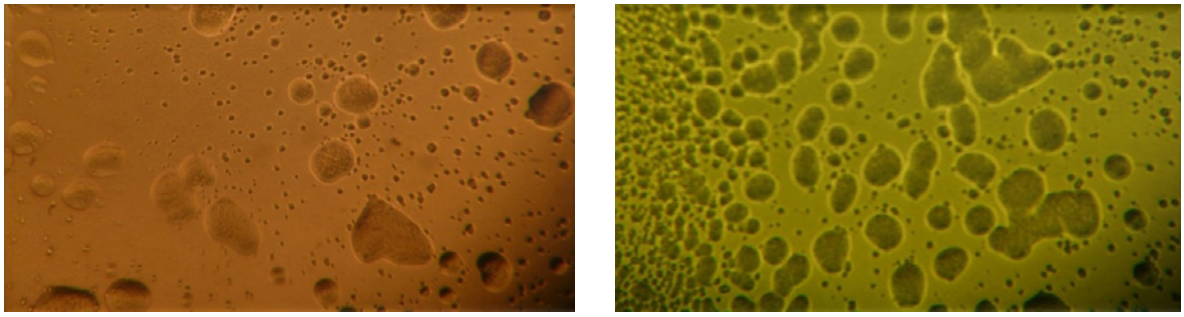

*Fig. 1. Mycoplasma gallisepticum colonies, grown on the cell-free nutrient medium supplemented with the bovine blood serum (magnification 200×)*

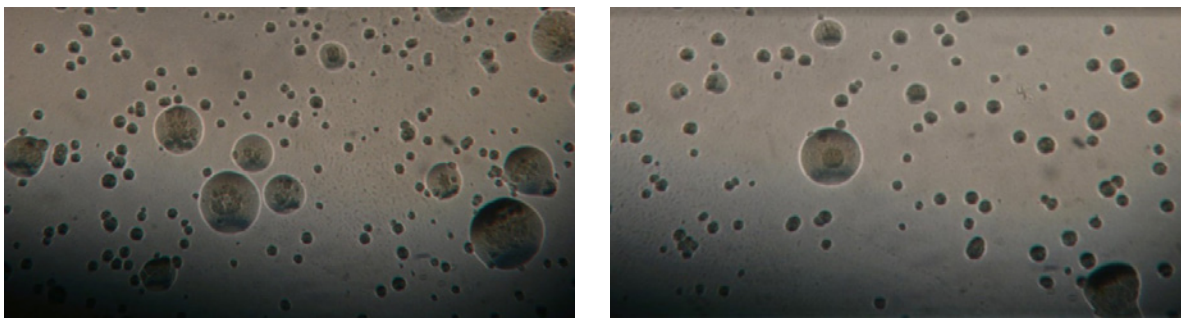

*Fig. 2. Mycoplasma gallisepticum colonies, grown on the cell-free nutrient medium supplemented with the porcine blood serum (magnification 200×)*

**Table 2**
**Growth properties of the cell-free nutrient medium supplemented with the bovine blood serum for *Mycoplasma synoviae* cultivation**

| Cell-free nutrient medium samples | Cattle blood serum contents (%) | Biomass growth rate (h), passage 1–3 | Biomass growth rate (h), passage 1–3 after cloning | Biomass growth rate (h), passage 1–3 after recloning | Culture activity at passage 9 in HA test (HAU $log_2$) | Growth properties at passage 9 (CFU/$cm^3$) |
|---|---|---|---|---|---|---|
| 1 | 12 | 140–144 | 115–120 | 94–96 | 2.0 | $10^3$ |
| 2 | 15 | 140–144 | 115–120 | 94–96 | 2.5 | $10^3$ |
| 3 | 20 | 138–140 | 114–120 | 92–94 | 3.0 | $10^4$ |
| 4 | 25 | 138–140 | 112–120 | 90–94 | 3.0 | $10^4$ |
| Control (cell-free nutrient medium with pig serum) | 12 | 78–96 | 78–88 | 78–88 | 5.0 | $10^6$ |

of view of the microscope are unevenly distributed, their morphology is polymorphic, there are oval, pear-shaped, poorly formed colonies of different sizes, the center is not pronounced or occupies most of the colony.

Figure 2 shows colonies of *Mycoplasma gallisepticum* grown on a nutrient medium supplemented with the porcine blood serum. Despite the colonies varying in size, round shapes with smooth edges and a distinctly formed and denser optical center predominate, which gives them the "fried egg" appearance characteristic of mycoplasmas.

The results of studies on the growth properties of the cell-free nutrient medium, which includes bovine blood serum, for growing *Mycoplasma synoviae* showed that the cultivation time is at least 140 hours at a serum concentration in the medium of 12 and 15% and 138 hours at a concentration of 20–25% (Table 2). When accumulating the biomass of *Mycoplasma synoviae*, it should also be taken into account that this type of mycoplasma belongs to difficult-to-cultivate microorganisms and has a lower adaptive capacity compared to *Mycoplasma gallisepticum*.

Similar to the results obtained in the study of the growth properties of the cell-free nutrient medium for the cultivation of *Mycoplasma gallisepticum*, when using the *Mycoplasma synoviae* clone, a reduction in the cultivation time to 96 hours was observed at passages 7–9. The agglutinating activity of *Mycoplasma synoviae* culture and the concentration of viable cells increased with an increase in the concentration of bovine blood serum in the medium from 12 to 20% and were in the range from 2.0 to 3.0 $log_2$ and $10^3$–$10^4$ CFU/$cm^3$, respectively. At the same time, the activity of the culture grown on Frey's medium with porcine blood serum (12%) was 5.0 $log_2$, and the CFU value was $10^6$ per 1 $cm^3$, which reliably indicates the clear advantages of using porcine blood serum in the cell-free nutrient medium for cultivating *Mycoplasma synoviae*.

Figure 3 shows the result of cultivating *Mycoplasma synoviae* on dense cell-free nutrient medium supplemented with the bovine blood serum (day 6 of culturing). The grown colonies are small in size, their diameter does not exceed 0.1 mm, and there is no optically dense center in their structure. The micrograph of Figure 4 shows colonies of *Mycoplasma synoviae* grown on a medium with porcine blood serum (6 days after seeding). Colonies are characterized by the appearance of "fried eggs", they are larger – up to 0.2 mm in diameter.

Thus, the results of the tests performed indicate that the growth properties of nutrient media for the cultivation of pathogenic mycoplasmas depend on the type of blood serum in their composition. It was noted that an increase in its concentration stimulated the growth of biomass with a reduction in the cultivation time. The use of porcine blood serum in the composition of the nutrient medium

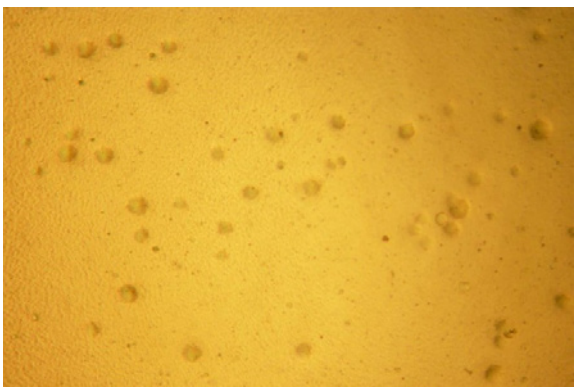

*Fig. 3. Mycoplasma synoviae grown on the cell-free nutrient medium supplemented with bovine blood serum (magnification 200×)*

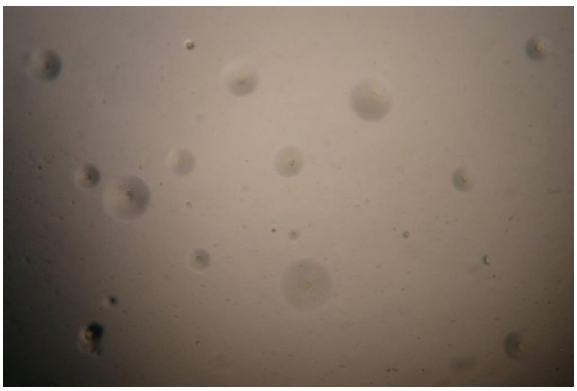

*Fig. 4. Mycoplasma synoviae grown on the cell-free nutrient medium supplemented with porcine blood serum (magnification 200×)*

at a concentration of 12% provided a more active biomass both in terms of the concentration of viable cells and the hemagglutinating and agglutinating activity of the culture with a minimum cultivation time of 78–96 hours.

To establish the reason for the low growth properties of the nutrient medium with the addition of cattle blood serum, a comparative analysis of some biochemical parameters of bovine and porcine blood serum was performed (Table 3) [18].

Despite the fact that the bovine and porcine blood serum contains a comparable amount of cholesterol, cattle have a large amount of high-density lipoproteins, characterized by a significantly low content of cholesterol and phospholipids [19]. Thus, serum with a high content of these lipoproteins may not meet the needs of mycoplasmas in fatty acids and cholesterol. On the contrary, the blood serum of pigs contains mainly low-density lipoproteins, which actively carry cholesterol and fatty acids, which can partially precipitate without any external influence. It is known that the causative agents of mycoplasmoses need these components, and the porcine blood serum makes up for the need for these compounds when cultivating mycoplasmas on the free-cell nutrient medium [14, 20].

Also, from the presented data, it can be seen that the carotene content in the bovine blood serum exceeds its concentration in the porcine blood serum from 5 to 20 times, and retinol – 3 times. Given that the components in question are the strongest antioxidants, they can slow down oxidative processes in cells, thereby increasing the interphase period or slowing down the process of accumulating resources for normal cell division, thereby initiating the appearance of non-viable mycoplasma cells. The close to zero carotene content in the porcine blood serum eliminates the risks associated with its potential impact on the growth and reproduction of mollicutes.

The results of the study indicate that the porcine blood serum is a significant growth component for the cultivation of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* and provides a more active biomass in the medium.

## CONCLUSION

As a result of the research, it was found that the cell-free nutrient medium supplemented with porcine blood serum has the most suitable growth characteristics for the optimal growth of *Mycoplasma synoviae* and *Mycoplasma gallisepticum* culture. The medium supplemented with bovine serum can also be used for the cultivation of these species of mollicutes, but it does not provide the same growth properties as the cell-free nutrient medium with porcine blood serum.

The low growth properties of the medium prepared using bovine blood serum are most likely associated with its biochemical composition, which contains 5–20 times more provitamin A than porcine blood serum, and cholesterol is mainly represented by high density lipoproteins. On the contrary, in the porcine blood serum, most of the lipoproteins have a low density, containing a large amount of fatty acids and cholesterol, which are the main structural elements of mycoplasma cells, in particular for the cytoplasmic membrane, which ensure its fluidity. The high content of carotene in the blood serum of cattle can increase the period of interphase during the reproduction of mycoplasmas.

**Table 3**
**Biochemical parameters of the bovine and porcine blood sera**

| Parameters | Bovine blood serum | Porcine blood serum |
|---|---|---|
| Serum cholesterol: | | |
| mg/100 mL | 50–170 | 60–110 |
| mmol/L | 1.30–4.42 | 1.56–2.86 |
| Serum carotene: | | |
| μg/100 mL | 500–2,000 | 0–10 |
| mg/L | 5.0–20.0 | 0–0.1 |
| Serum Vitamin A: | | |
| μg/100 mL | 30–90 | 10–35 |
| μmol/L | 1.05–3.14 | 0.35–1.22 |

Twelve percent concentration of porcine blood serum in the cell-free nutrient medium is optimal for preparing a culture with high biological activity with a short cultivation time (72–96 hours).

Adaptation of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* to the cell-free nutrient medium supplemented with the bovine blood serum prior to passage 9 did not allow preparing a biomass with an activity similar to that of the medium with porcine blood serum, which should be taken into account when cultivating these species of mollicutes on a large scale.

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