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Development of submerged cultivation method for vaccine *Mycoplasma mycoides* subsp. *mycoides* strain

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SUMMARY

Members of *Mycoplasma* genus are widely spread in nature (soil, water, manure, cereals, food products), and there are ones pathogenic for humans, animals and birds. The group of highly dangerous diseases include contagious bovine pleuropneumonia (CBPP) caused by *Mycoplasma mycoides* subsp. *mycoides*. Risk of the disease agent introduction to Russia with imported livestock and raw materials still remains. Therefore, of topical importance is the improvement of the CBPP vaccine manufacturing technology. The studies were aimed at the development of the method of the submerged cultivation of the vaccine *Mycoplasma mycoides* subsp. *mycoides* strain. This method allows production of a large amount of the biological material and simplification of the biological product manufacturing technology. Dynamics of the mycoplasma mass accumulation during the submerged cultivation was examined within the studies. Four phases of the bacterial growth were clearly demonstrated. Insignificant decrease of the microbial cell concentration was reported in the first two days of cultivation; days 3 and 4 were specified by the increase of the microbial mass concentration by several orders of magnitude: from 2.5×10^8 to 4.5×10^9 cells/volume unit, on day 5 the concentration was in equilibrium and starting from day 6 the onset of the microorganism's death phase was reported. Similar dynamics was demonstrated during cultivation in the bioreactors. Singular fried egg-shaped colonies or their accumulations were observed at the visual examination of the submerged cultivated mycoplasma. Therefore, when using submerged cultivation method and such parameters as mycoplasma seeding at a dose of 10^5 microbial cells / volume unit; 2/3 filling volume; incubation at (37 ± 0.5) °C; agitation at 90 rpm and use of synthetic nutrient medium, the bacterium accumulates at the titre of 10^9 cell/volume unit.

Keywords: *Mycoplasma mycoides* subsp. *mycoides*, contagious bovine pleuropneumonia, cultivation, growth phases

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Разработка способа глубинного культивирования вакцинного штамма *Mycoplasma mycoides* subsp. *mycoides*

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РЕЗЮМЕ

Представители рода *Mycoplasma* широко распространены в природе (почве, воде, навозе, злаках, пищевых продуктах), среди них имеются виды, патогенные для человека, животных и птиц. К группе особо опасных инфекционных болезней отнесена контагиозная плевропневмония крупного рогатого скота, возбудителем которой является *Mycoplasma mycoides* subsp. *mycoides*. В настоящее время риск заноса возбудителя инфекции на территорию России с импортным скотом и сырьем из неблагополучных регионов сохраняется. В связи с этим совершенствование технологии изготовления вакцины против контагиозной плевропневмонии является актуальной задачей. Целью данного исследования явилась разработка глубинного способа культивирования вакцинного штамма *Mycoplasma mycoides* subsp. *mycoides*. Данный метод позволяет получать за короткий промежуток биомассу в больших объемах и упрощает технологию изготовления биопрепаратов. В процессе работы изучена динамика накопления биомассы микоплазмы при безопорном методе культивирования. Наглядно продемонстрированы 4 фазы роста бактерии. В первые двое суток выращивания отмечали незначительное снижение концентрации микробных клеток, третьи – четвертые сутки характеризовались увеличением биомассы на несколько порядков от $2,5 \times 10^8$ до $4,5 \times 10^9$ клеток в единице объема, на 5-е сутки концентрация находилась в равновесном положении, и начиная с 6-х суток регистрировали наступление фазы гибели микроорганизма. Аналогичная динамика прослеживалась и при культивировании в биореакторе. При визуализации микоплазмы, полученной при

выращивании глубинным способом, на твердой питательной среде наблюдали единичные колонии или их скопления, имеющие вид яичницы-глазуньи. Таким образом, используя безопорный метод культивирования и такие параметры, как засев микоплазмы в дозе 10^5 микробных клеток в единице объема, объем заполнения на 2/3, температура инкубирования (37 ± 0,5) °С, перемешивание при 90 об/мин, а также применение синтетической питательной среды бактерия накапливается в титре 10^9 клеток в единице объема.

Ключевые слова: *Mycoplasma mycoides* subsp. *mycoides*, контагиозная плевропневмония крупного рогатого скота, культивирование, фазы роста

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INTRODUCTION

Large amount of the submerged cultivated biomass is used in the manufacture of the anti-bacterial products. The method of the submerged cultivation was put into practice by N. E. Lebedev in 1950. The scientist demonstrated the possibility of *Enterobacteriaceae* family members' cultivation in the bioreactors with forced aeration and agitation [1].

This method allowed for significant achievements in the development of the industrial-scale biological product manufacturing technologies [2]. Growth technologies of tularemia bacteria [3], *Bacillus subtilis* and *Bacillus licheniformis* [4] used for probiotics production, etc. were, *inter alia*, improved.

When submerged cultivated the microbial cells are grown in liquid nutrient medium in special equipment – bioreactors of different modifications. The reservoir is equipped with the agitator of various configurations with adjusted rotation speed, sensors of temperature, pO_2, pH and foam levels, whose readings are transmitted to the computer display. Outside the bioreactor there are sampling ports and other devices [5].

Liquid nutrient media are used for submerged cultivation. The media in such physical condition facilitate access of the bacteria to the nutrient substances; they are easily mixed when incubated thus allowing for the replenishment of the nutrients essential for the culture [6].

Such bacteria as mycoplasmas are highly sensitive to the growth media. In natural environment they are in close contact with the host cells thus readily receiving all substances necessary for their development. The surface membranes of the mycoplasma and body cells are nearly similar in structure, therefore, close membrane contact occurs by way of dissolving in each other. This interaction facilitates transportation of the nutrients essential for mycoplasma from the host cell cytoplasm. Such cooperation resulted in the evolutionary loss by the microorganism of the genes coding these substances. Therefore, artificial nutrient media for mycoplasma cultivation shall contain complex components necessary for their growth. The protein base of the medium is the beef heart extract or peptone. Back in the 1970s, the mostly recognized media included media based on Martin's peptone and bovine heart broth. Yeast extract is used as the growth factor and source of vitamin B. The source of sterol is the equine serum (less frequently, porcine one), supplemented in large amounts. Bovine serum is not used as it can inhibit growth of some mycoplasma species [7]. Thus, porcine serum is used for *Mycoplasma gallisepticum* and *Mycoplasma synoviae* cultivation as it allows production of a large amount of the mycoplasma biological material in the modified Frey's medium [8]. The source of energy involves glucose, L-arginine or urea [9].

The majority of mycoplasma are facultative parasites or commensals of animals and plants. The objects of the mycoplasma paratization are quite numerous starting from plants and up to mammals (including humans). There are mycoplasma species pathogenic for humans, animals and birds. They include *M. mycoides* – agent of bovine, sheep and goat pneumonia; *M. agalactiae* – agent of rheumatoid syndrome in sheep and goats, *M. pneumoniae* – agent of acute respiratory diseases and primary atypical pneumonia in humans, which demonstrates high hemolytic activity [10].

Among all mycoplasmas, *Mycoplasma mycoides* subsp. *Mycoides* should be specifically mentioned as it causes such highly contagious bovine disease as contagious pleuropneumonia. The disease is characterized by fever, fibrinous interstitial pneumonia, serofibrinous pleurisy followed by formation of anemic necrosis and sequestra in the lungs and accumulation of large amounts of exudate in the thoracic cavity [11–13].

The global community rates the disease as a highly dangerous one, and it is included in the list of notifiable diseases by the World Organization for Animal Health (WOAH). The disease is transboundary as well, and its spread can result in grave consequences at the national level. Annual economic losses due to contagious pleuropneumonia suffered by the African countries amount to 30 mln euros [14–16]. In the late XIX – early XX centuries,

the disease prevailed in European, Asian, African countries and in Australia. Severe animal health measures implemented in different countries allowed for the disease eradication in many localities of the globe. In the natural environment the abovementioned pathogen infects only ruminants, mostly cattle and zebu. In cattle contagious pleuropneumonia is manifested by changed general health condition (loss of appetite, pyrexia) and respiratory signs (dyspnoea, polypnea, coughing and nasal discharge). Sub-acute and acute disease is most often reported. The animals pose the highest risk of the infection transmission in case of chronic disease lacking obvious clinical signs [17].

The Russian Federation is the first EAEU country, which in 2020 was included in the WOAH list of 20 countries officially free from bovine contagious pleuropneumonia (there are total of 182 WOAH member countries). The key importance is currently given to the prevention of the disease agent introduction into the country with the animals and raw materials imported from the infected regions [18].

In this context, the vaccine production technology should be developed, and there are critical control points during the production process. Such enriched nutrient media as F_{66} and Gourlay are used for mycoplasma cultivation that in turn leads to possible bacterial contamination (unintentional contamination). Use of antibiotics during the medium preparation is very complicated due to high sensitivity of mycoplasma to them (apart from penicillins and sulfanilamides). This microorganism is demanding to the medium pH, thus, hydrogen ion exponent should be set within 7.8–8.0. The pH is rapidly decreasing during cultivation that affects the mycoplasma harvest and its viability outside the cultivation environment [18].

Therefore, there are a number of difficulties in the mycoplasma cultivation procedure, which should be given particular attention; and use of submerged cultivation in biotechnology is of topical importance.

The study was aimed at the development of the submerged cultivation method for *Mycoplasma mycoides* subsp. *mycoides*.

MATERIALS AND METHODS

Mycoplasma mycoides subsp. *mycoides* "MA-VNIIVVIM" strain was used in the study. The strain was received from the State collection of microorganisms causing dangerous or highly dangerous diseases, including zooanthroponoses, and exotic animal diseases (CCP register number – 441429)[1].

Nutrient media: nutrient broth and nutrient agar (Oxoid Ltd., Great Britain), yeast extract (Sanofi Diagnostics Pasteur, France) and equine serum (BioloT Ltd., Russia). Nutrient media were prepared according to the manufacturer's instructions. The media were sterilized by autoclaving at 121 °C for 15 min.

Equipment: bioreactor, 5 L (NBS, USA), shaker. In incubator shaker, cultivation was performed in 250 cm^3 Erlenmeyer flasks with 50 cm^3 of the nutrient medium. Key parameters were automatically controlled during cultivation in bioreactor: temperature, agitation speed, pH of the media.

Cultivation in flasks in incubator shaker. The nutrient medium was based on liquid broth and 10% yeast extract. The medium was supplemented with 20% of inactivated equine serum and filled at 50 cm^3 in 250 cm^3 flasks. The bacterial inoculum was reconstituted to the initial volume and 10-fold dilutions (10^{-1} and 10^{-2}) were prepared. Then 5.0 cm^3 of the 10^{-2} inoculum were transferred to the ready nutrient medium. The flasks with the infected material and control flask were incubated at (37 ± 0.5) °C and static shaking of 100 rpm.

Cultivation in bioreactor. The ready nutrient medium (3 L) was infected with working inoculum (300 cm^3) and transferred to the closed system bioreactor. Automated cultivation mode was set: temperature – (37 ± 0.5) °C, agitation – 90 rpm, bubble aeration followed by agitation – from day 2 of cultivation.

Upon the cultivation completion, the cell viability was assessed, the microorganism was visualized and microbiological purity of the culture was determined.

The microbiological purity was controlled by inoculation into the nutrient media according to GOST 28085-2013[2].

The viable cell concentration was determined by endpoint dilution method. Pooled sample was used to prepare the ten-fold dilutions (10^{-1}–10^{-9}) with phosphate buffered solution (pH 7.2–7.4). Then 1.0 cm^3 of each dilution starting from 10^{-4} and up to 10^{-9} were inoculated in 3 tubes with ready nutrient medium. The tubes were incubated at (37 ± 0.5) °C for 14 days. After that presence or absence of the microorganism growth was visually recorded by the opalescence appearance. The most probable number of the cells in the volume unit was calculated according to McCredy's table.

The strain was visualized by inoculating 0.1 cm^3 of each endpoint dilution demonstrating well-expressed opalescence in the broth into the dishes with solid synthetic nutrient medium. After the complete absorption, the dishes with the inoculates were transferred to the CO_2-incubator. The inoculates were incubated for 5–7 days at (37 ± 0.5) °C in the 5% CO_2 environment at 95% relative humidity. The resulted colonies grown on the solid media were microscopically examined using Opton ID 03 optical microscope (ZEISS, Germany).

RESULTS AND DISCUSSION

During the first round of the experiments, the dynamics of mycoplasma material accumulation was determined when cultivated in the suspension containing flasks in the shaker. The cultivation was proceeded until the culture reached the stationary growth phase. The initial mycoplasma concentration in the nutrient medium at inoculation was determined as 11.5×10^6 of the most probable number of microbial cells per volume unit. Hereafter, during the subsequent 7 days of cultivation, samples were collected for the assessment of the biological material accumulation. The results are demonstrated in Figure 1.

The graph demonstrates that the lag phase continued for 2 days of growth, when insignificant decrease of the microbial cell concentration was observed and the titres amounted to 2.5×10^5 – 9.5×10^5. Day 3 and 4 were viewed as logarithmic growth phase when bacterial population increased by several orders of magnitude.

[1] State collection of microorganisms causing dangerous or highly dangerous diseases, including zooanthroponoses, and exotic animal diseases. Available at: https://ckp-rf.ru/catalog/ckp/441429.

[2] GOST 28085-2013 Medicine remedies biological for veterinary use. Method of bacteriological control of sterility. Available at: https://meganorm.ru/Data2/1/4293775/4293775115.pdf.

During this period, the mycoplasma accumulated up to 2.5×10^8 – 4.5×10^9 cells per volume unit. On day 5, the concentration was in equilibrium with the log phase and amounted to 4.5×10^9 being indicative of the transmission of the culture to the stationary growth phase. Starting from day 6 of cultivation, the significant decrease of the bacterial material was observed that specified the onset of the death phase.

The results of the experiment, therefore, demonstrated that during the submerged cultivation the mycoplasma passes through all four growth phases. The maximal cell accumulation in the volume unit was observed on day 4 of cultivation during the exponential growth phase, the duration of which amounted to 48 hours for the tested strain.

Optimal pH levels of the medium used for *Mycoplasma mycoides* subsp. *mycoides* "MA-VNIIVVIM" strain propagation ranged from 7.8 to 8.0. Changes of pH according to the oncoming growth phase were reported during cultivation in flasks. Within the first 2 days, the medium acidity matched the original pH level of the prepared cultivation medium and equaled 8.0. On day 3–4, the pH level amounted to 7.8. Upon the new phase onset, the pH level gradually declined thus being indicative of active biomass accumulation. On day 5–6, the pH varied from 7.7 to 7.5 that was associated with the accumulation of bacteria waste products and decrease of nutrient substances essential for the growth of the microorganism, which in turn affected the culture growth.

Therefore, during the mycoplasma cultivation the hydrogen ion concentration in the cultivation medium changes depending on the bacterial growth phase.

The parameters selected for cultivation in flasks (filling level, infection dose, cultivation duration, agitation speed) were applied for propagation in 5-liter bioreactor. During cultivation the major attention was given to the babbling as the nutrient medium contained high percentage of serum that could result in foam formation. The results of the studies of the microbial cell accumulation dynamics are shown in the table.

As you can see, in the bioreactor all bacterial growth phases can be traced, which are typically observed during cultivation in flasks in the shaker. On day 4 of cultivation, high accumulation of the mycoplasma was reported – 2.8×10^9 cell/ volume unit. The duration of the exponential growth phase in the bioreactor also amounted to 48 hours with equal accumulation of the biomass.

Therefore, the submerged cultivation method can be used for the bacteria propagation during the contagious bovine pleuropneumonia vaccine production.

To visualize the mycoplasma produced by the submerged cultivation method, the tested sample was inoculated onto the solid nutrient medium. On day 5 of cultivation, the grown mycoplasma colonies were well seen by microscopy. Both singular colonies and their accumulation were in sight (Fig. 2).

The colonies were of regular round saucer-like shape with yellowish core and transparent gray edges (Fig. 3). There were colonies in the stage of division by budding (Fig. 4).

Hence, the biomass with high accumulation rate was obtained during the submerged cultivation, and when inoculated onto the solid nutrient media the mycoplasma had typical fried egg-shaped appearance.

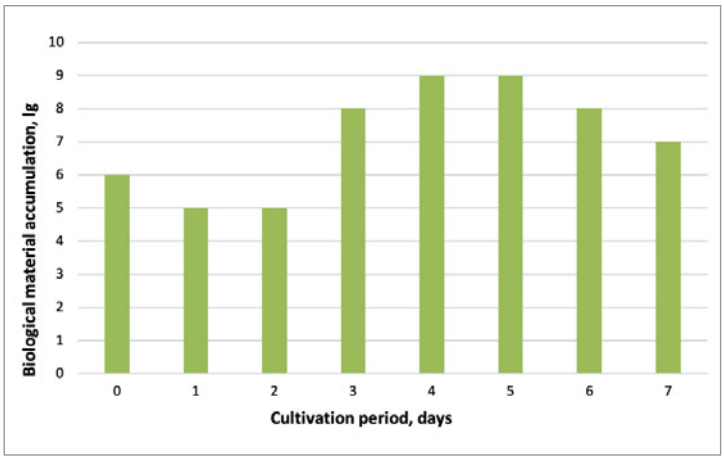

Fig. 1. Dynamics of mycoplasma population growth at suspension cultivation

Table
Accumulation of mycoplasma vaccine strain "MA-VNIIVVIM" in the fermenter

	Cultivation duration (day)			
	0	2	3	4
Activity (MPN)	9.5×10^5	2.5×10^4	9.5×10^8	2.8×10^9

MPN – most probable number.

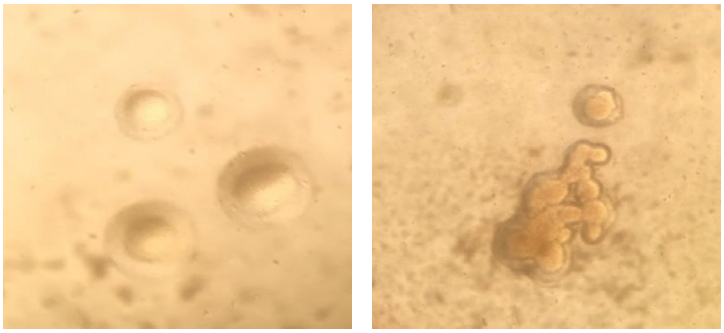

Fig. 2. Mycoplasma colonies

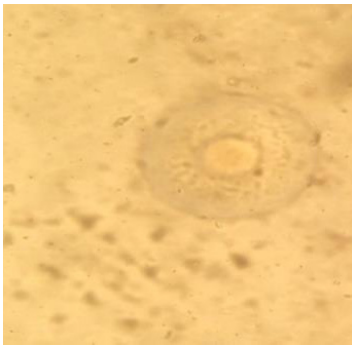

Fig. 3. Colony center

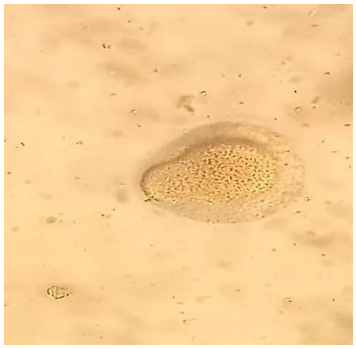

Fig. 4. Colony in the process of replication

As well as continuous cell cultures, the bacteria, and mycoplasmas in particular, are suspension cultivated and pass through all growth cycle phases [19]. The mycoplasmas are cultivated for a relatively long time in contrast to other bacteria, whose cultivation can take 18 hours, e.g. salmonella [20].

When mycoplasma is cultivated in suspension, the growth process is clearly demonstrated both in shaker and in bioreactor. Each of four stages took a certain time period. It was determined that loss of the bacterial mass by one order of magnitude was observed in the lag-phase, which continued for two days. Exponential, or log-phase, also lasted for two days as distinct from other bacteria, which need from several minutes to 24 hours for the phase. For instance, in case of salmonellas this phase takes 20–30 min; coliforms – 15–17 min, staphylococcus – 25–35 min, bacillus Kochii – 19–20 hours [19]. The stationary phase, during which equilibrium between the bacterial growth and death was observed, lasted for one day and the death phase further occurred.

Use of bioreactor for the mycoplasma cultivation allowed to automatize the key cultivation parameters: temperature, agitation of the liquid and air phases. Due to continuous agitation all components of the medium as well as microbial cells were evenly distributed all over the working space of the bioreactor thus allowing for homogenous bacterial mass production [21].

CONCLUSION

The study results demonstrated that submerged cultivation method can be used for mycoplasma biomass production. Such cultivation parameters as mycoplasma seeding at 10^5 microbial cells/volume unit, filling at 2/3 of the volume, incubation temperature (37 ± 0.5) °C, agitation at 90 rpm and use of synthetic nutrient medium resulted in the bacterium accumulation at the titre of 10^9 cells/volume unit at logarithmic growth stage.

It was determined that when submerged cultivated the vaccine strain *Mycoplasma mycoides* subsp. *mycoides* "MA-VNIIVVIM" follows routine patterns during the four phases of the growth cycle. Exponential phase duration amounted to 48 hours. In case of this cultivation method the strain retained the mycoplasma-typical growth properties: distinct opalescence in the liquid synthetic medium and development of the pronounced fried egg shape when inoculated onto the solid synthetic nutrient medium.

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