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Optimization of medium composition and study of growth stages of *Mycoplasma bovis* "Kaluga 2020" isolate

Mohammad Abed Alhussen[1], A. A. Nesterov[2], A. V. Sprygin[3], I. N. Shumilova[4], M. S. Bryantseva[5], O. P. Byadovskaya[6]

[1] People's Friendship University of Russia (RUDN University), Moscow, Russia

[2–6] FGBI "Federal Centre for Animal Health" (FGBI "ARRIAH"), Vladimir, Russia

[1] *https://orcid.org/0000-0002-1210-0303, e-mail: alhussenmohammed85@hotmail.com*

[2] *https://orcid.org/0000-0002-4288-1964, e-mail: nesterov@arriah.ru*

[3] *https://orcid.org/0000-0001-5982-3675, e-mail: sprygin@arriah.ru*

[4] *https://orcid.org/0000-0001-6132-5771, e-mail: shumilova@arriah.ru*

[5] *e-mail: bryantseva@arriah.ru*

[6] *https://orcid.org/0000-0002-8326-7151, e-mail: bjadovskaya@arriah.ru*

SUMMARY

Mycoplasma bovis is considered one of bovine mycoplasmosis pathogens responsible for respiratory diseases, mastitis, arthritis and keratoconjunctivitis. The paper presents results of the study on optimizing the component composition of the culture medium for *Mycoplasma bovis* "Kaluga 2020" isolate, as well as the study of this pathogen's growth stages. The color-changing units assay and the culture method combined with colony-forming unit quantification were used for determination of *Mycoplasma* activity. It was found that when cultured in an optimized nutrient medium based on modified Hayflick broth, the microorganism enters a logarithmic growth phase after first 24 hours of growth, in 72 hours the *Mycoplasma* culture enters a stability phase, and a decline phase is recorded in 84 hours. The effect of percentage content of glucose, fresh yeast extract and horse serum in the nutrient medium on accumulation of *Mycoplasma bovis* "Kaluga 2020" isolate was evaluated using the one-factor-at-a-time approach. It was found that the greatest effect on *Mycoplasma* accumulation was exerted by such growth factors as fresh yeast extract and horse serum in the nutrient medium ($p < 0.05$), while changes in the amount of glucose did not stimulate *Mycoplasma bovis* growth. Based on results of the conducted studies, the appropriate composition was determined and the optimal content of growth factors in the medium for culturing *Mycoplasma bovis* "Kaluga 2020" isolate was selected: 12.5% of fresh yeast extract and 25% of horse serum. The use of the optimized nutrient medium based on modified Hayflick broth allowed 5-fold increase in accumulation of *Mycoplasma* biomass (3.98×10^9 CFU/ml) compared to the standard medium (0.79×10^9 CFU/ml).

Keywords: *Mycoplasma bovis*, cattle, "Kaluga 2020" isolate, optimization, nutrient media, colony-forming units (CFU), color-changing unit (CCU), biological activity

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For correspondence: Mohammad Abed Alhussen, Post-Graduate Student, Department of Veterinary Medicine, Agrarian and Technological Institute, People's Friendship University of Russia (RUDN University), 117198, Russia, Moscow, ul. Miklukho-Maklay, 6, *e-mail: alhussenmohammed85@hotmail.com*.

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Оптимизация состава питательной среды и изучение стадий роста изолята «Калуга 2020» *Mycoplasma bovis*

Мохаммад Абед Алхуссен[1], А. А. Нестеров[2], А. В. Спрыгин[3], И. Н. Шумилова[4], М. С. Брянцева[5], О. П. Бьядовская[6]

[1] ФГАОУ ВО «Российский университет дружбы народов» (РУДН), г. Москва, Россия;

[2–6] ФГБУ «Федеральный центр охраны здоровья животных» (ФГБУ «ВНИИЗЖ»), г. Владимир, Россия

[1] *https://orcid.org/0000-0002-1210-0303, e-mail: alhussenmohammed85@hotmail.com*

[2] *https://orcid.org/0000-0002-4288-1964, e-mail: nesterov@arriah.ru*

[3] *https://orcid.org/0000-0001-5982-3675, e-mail: sprygin@arriah.ru*

[4] *https://orcid.org/0000-0001-6132-5771, e-mail: shumilova@arriah.ru*

[5] *e-mail: bryantseva@arriah.ru*

[6] *https://orcid.org/0000-0002-8326-7151, e-mail: bjadovskaya@arriah.ru*

РЕЗЮМЕ

Mycoplasma bovis является одним из возбудителей микоплазмозов крупного рогатого скота, вызывающим респираторные болезни, маститы, артрит и кератоконъюнктивит. В статье представлены результаты исследования по оптимизации компонентного состава питательной среды для культивирования изолята «Калуга 2020» *Mycoplasma bovis*, а также изучения стадий роста возбудителя. Для определения активности микоплазм использовали метод измерения цветоизменяющих единиц и культуральный метод с подсчетом колониеобразующих единиц. Установлено, что при культивировании в оптимизированной питательной среде на основе модифицированного бульона Хейфлика микроорганизм вступает в фазу логарифмического роста по истечении первых 24 ч роста, через 72 ч культура микоплазм переходит в стабильный период, а через 84 ч регистрируется фаза спада. Влияние процентного содержания глюкозы, свежего дрожжевого экстракта и сыворотки крови лошади в питательной среде на накопление изолята «Калуга 2020» *Mycoplasma bovis* оценивали с использованием метода «один фактор за раз». Было установлено, что наибольшее влияние на накопление микоплазм оказывало содержание в питательной среде таких факторов роста, как свежий дрожжевой экстракт и сыворотка крови лошади ($p < 0,05$), в то время как изменение количества глюкозы не стимулировало рост *Mycoplasma bovis*. В результате проведенных исследований определен подходящий состав и подобрано оптимальное содержание факторов роста в среде для культивирования изолята «Калуга 2020» *Mycoplasma bovis*: 12,5% свежего дрожжевого экстракта и 25% сыворотки крови лошади. Применение оптимизированной питательной среды на основе модифицированного бульона Хейфлика позволило увеличить накопление биомассы микоплазм в 5 раз ($3,98 \times 10^9$ КОЕ/мл) по сравнению со стандартной средой ($0,79 \times 10^9$ КОЕ/мл).

Ключевые слова: *Mycoplasma bovis*, крупный рогатый скот, изолят «Калуга 2020», оптимизация, питательные среды, колониеобразующие единицы (КОЕ), цветоизменяющая единица (ЦИЕ), биологическая активность

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INTRODUCTION

Mycoplasma bovis (*M. bovis*) is a causative agent of bovine mycoplasmoses, which is widespread all over the world and is common in the Russian Federation as well [1, 2]. This pathogen is one of the etiological agents of bovine respiratory diseases, also causing mastitis, arthritis and keratoconjunctivitis [3, 4].

M. bovis was first isolated from cattle with severe mastitis in the USA in 1961 [5, 6]. *M. bovis* is considered responsible for a quarter to a third of all economic losses in cattle industry due to respiratory diseases [7].

Laboratory diagnosis of bovine mycoplasmoses includes cultural, serological and molecular test methods [8, 9]. At the same time, the pathogen isolation by culturing in nutrient media is one of the most reliable methods of the disease diagnosis. Currently, various types of nutrient media are widely used for *M. bovis* cultivation, including Hayflick medium [10], modified PPLO medium [11], Eaton's medium [12] and others.

Optimization of nutrient medium composition is one of the most important aspects for improving the mycoplasma culture technique, as well as for diagnostic studies using isolation method [13]. At the same time, the complexity of the component composition of nutrient media and the long period of mycoplasma growth require multistage studies [14].

Mycoplasma growth rate and activity are estimated using several methods: color-changing unit (CCU) assay, colony-forming unit (CFU) count, measurement of turbidity, reduction of tetrazolium salts to formazane, determination of adenosine triphosphate (ATP) cell concentrations using luciferin-luciferase luminometry, etc. [15].

The aim of the paper is to study *M. bovis* growth dynamics during *in vitro* cultivation and to optimize the component composition of Hayflick nutrient medium.

MATERIALS AND METHODS

Isolate. M. bovis "Kaluga 2020" isolate recovered from biological material samples of calves demonstrating clinical signs of respiratory disease in 2020 was used for the study. The *M. bovis* isolate was identified using real-time polymerase chain reaction.

Nutrient media. Modified Hayflick broth was used as a standard liquid nutrient medium [16]. BBL™ Mycoplasma Broth Base (BD, USA) was used for its preparation. BBL™ Mycoplasma broth base in an amount of 20 g was dissolved in 1 L of distilled water, thoroughly mixed and sterilized by autoclaving at a temperature of (121 ± 0.5) °C for 15 minutes, then it was cooled to (50 ± 2) °C. 20 mL of non-heated horse serum, 0.5 mL of 40% glucose solution, 10 mL of fresh 25% yeast extract solution, 1.5 mL of 0.5% phenolic red solution, 1 mL of penicillin solution (200,000 units/mL) and 0.04 mL of 10% thallium acetate solution were added to 100 mL of the medium. The pH of the finished broth was adjusted to 7.8 by adding 1.0 M NaOH solution.

To prepare a solid nutrient medium, 6.7 g of Bacto™ Agar (BD, USA) was added to the modified Hayflick broth. After autoclaving, the semi-product was cooled to a temperature of (50 ± 2) °C, and medium supplements were

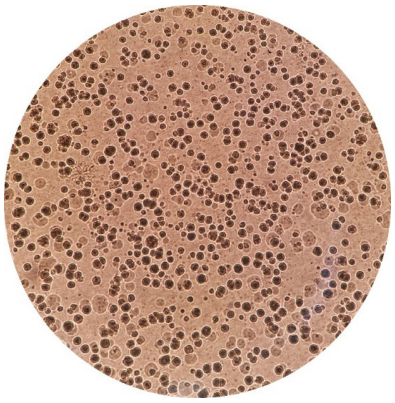


Fig. 1. 3-day-old M. bovis culture grown in solid nutrient medium (magnification 20×)

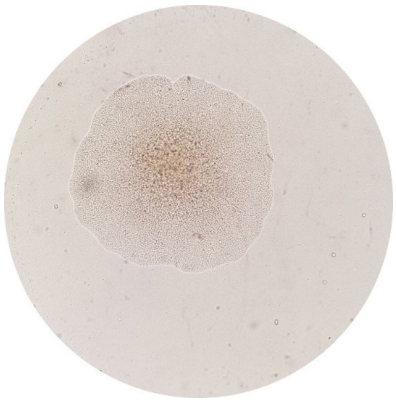


Fig. 2. 4-day-old M. bovis culture grown in solid nutrient medium (magnification 40×)

added according to the above-specified procedure. The prepared nutrient medium was poured into Petri dishes, cooled until completely solidified and stored at a temperature of (4 ± 2) °C.

Determination of M. bovis "Kaluga 2020" isolate activity. To determine the mycoplasma activity, the CCU assay and culture method with CFU count were used. A series of 10-fold successive dilutions of the suspension containing *M. bovis* "Kaluga 2020" isolate (10^{-1}–10^{-5}) were prepared in Hanks' saline solution. 10 µL of microbial suspension of each dilution were inoculated in solid nutrient medium in three duplicates. The inoculations were cultivated in a thermostat at a temperature of (37 ± 0.5) °C in 5% CO_2-enriched atmosphere for 9 days. Reading of titration results was conducted by counting single colonies and calculating the average CFU number in 10 µL of the highest suspension dilution, in which the growth of *M. bovis* colonies was observed. The obtained value was used to calculate the CFU number in 1 mL of the initial suspension of the test material.

The activity of *M. bovis* "Kaluga 2020" isolate was evaluated in 96-well culture plates using the color-changing units [17, 18] method. 20 µL of initial *M. bovis* suspension were mixed with 180 µL of modified Hayflick broth with phenolic red in the first wells of the plate, then serial 10-fold dilutions of the test suspension were prepared (10^{-1}–10^{-10}). Wells without *M. bovis* were used as a nutrient medium control. The plates were incubated at a temperature of (37 ± 0.5) °C in 5% CO_2-enriched atmosphere for 14 days. Accumulation of *M. bovis* metabolic products results in a pH shift to acidity, which causes a color change of the indicator from red to yellow. The color change of the nutrient medium and *M. bovis* activity in the culture suspension were recorded every 24 hours.

The concentration (titer) was determined in CCU/mL as the maximum dilution of *M. bovis*-containing suspension, in which a color change was observed [19].

The pH of the medium was monitored using a pH meter according to the operating instructions of the device.

Statistical analysis. The Minitab/Statistics program (version 19.1, USA) was used for experimental data analysis. The results obtained were found to be reliable ($p < 0.05$).

Table 1
Correlation between *M. bovis* biological activity and CCU value

Duration of cultivation, h	Medium color change	Mycoplasma biological activity		pH
		lg CFU/mL	lg CCU/mL	
24	from red to red-orange	7.0	5.0	7.4
72	from red-orange to orange	8.9	10.0	7.2
96	from orange to orange-yellow	8.0	9.0	7.1
168	from orange-yellow to yellow	6.4	4.0	6.8

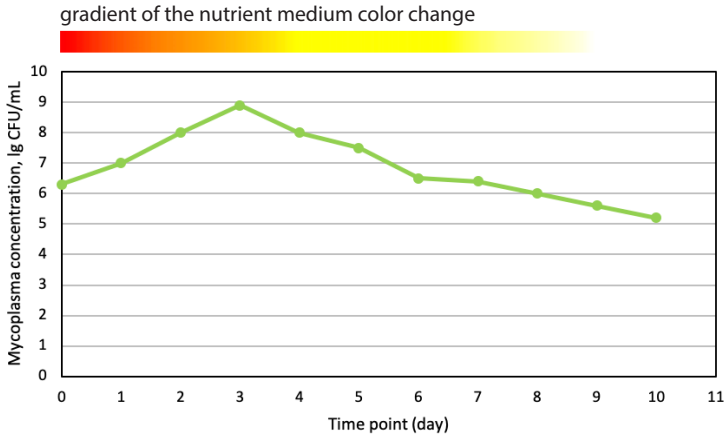


Fig. 3. Growth phases and accumulation of M. bovis "Kaluga 2020" isolate when cultivated in modified Hayflick broth

TEST RESULTS

During cultivation of *M. bovis* on solid modified Hayflick medium the colonies with irregular edges and a knobby protuberance resembling a fried egg were observed (Fig. 1, 2).

Determination of growth phases and maximum accumulation time of M. bovis "Kaluga 2020" isolate when cultivated in modified Hayflick broth. *M. bovis* "Kaluga 2020" isolate was cultivated at a temperature of (37 ± 0.5) °C for 10 days, while samples of culture suspension were taken every 24 hours and the *M. bovis* activity was estimated by titration methods using CFU count (culture method) and color-changing units assay. The study results showed that the "Kaluga 2020" isolate culture entered the logarithmic growth phase after 24 hours of cultivation and the maximum level of mycoplasma biomass

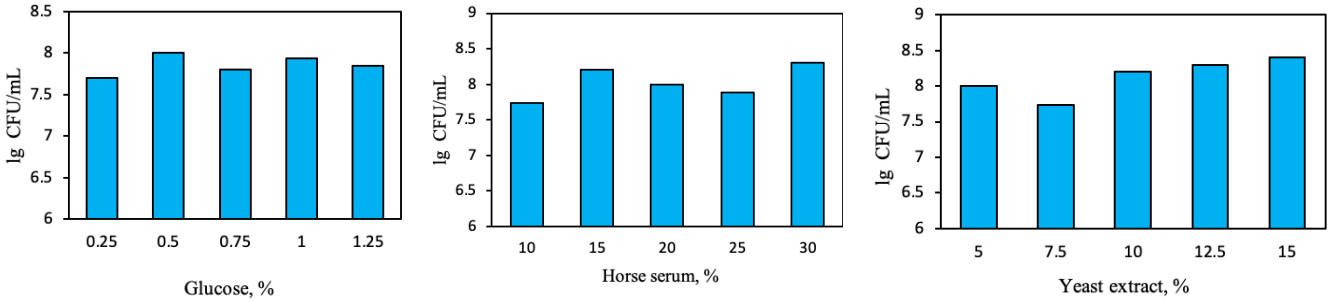


Fig. 4. Effect of various components of nutrient medium on accumulation of M. bovis "Kaluga 2020" isolate

accumulation was recorded in 72 hours, after which a decline phase occurred with a decrease in the biological activity of the cultivated material (Fig. 3).

Correlation of biological activity values of mycoplasmas identified by color-changing units assay and cultural method. Analysis of the obtained results showed that the maximum isolate accumulation was observed in 72 hours of cultivation (8.9 lg CFU/mL). The indicated mycoplasma concentration correlated with the change in the color of the nutrient medium (10 lg CCU/mL), which corresponded to a pH of 7.2. With longer cultivation, the biological activity of the cultured material was recorded to decrease to 6.4 lg CFU/mL, which was comparable to the concentration of microorganisms equal to 4 lg CCU/mL (Table 1).

Determination of the optimal nutrient medium composition for M. bovis "Kaluga 2020" isolate cultivation. At the first stage of the work, the modified Hayflick medium components that most significantly affected the accumulation of *M. bovis* "Kaluga 2020" isolate were determined. The studies were carried out by modifying the standard composition of the Hayflick medium with respect to the three components: glucose, fresh yeast extract and horse serum. At the same time, the percentage quantity of only one of the three components was changed in each series of experiments, while the standard parameters of other growth factors remained the same.

To determine the effect of glucose on *M. bovis* growth, a nutrient medium containing this component at 0.25; 0.50; 0.75; 1.00; 1.25% was used, fresh yeast extract – at 5.0; 7.5; 10.0; 12.5; 15.0%, horse serum – at 10, 15, 20, 25, 30%. Changes in the biological activity of mycoplasmas were monitored by CFU count using titration method.

The obtained results showed that the content of two growth factors in the nutrient medium is of the highest importance for *M. bovis* accumulation: fresh yeast extract and horse serum, while glucose does not have a significant effect (Fig. 4).

After identification of the most significant growth factors of the nutrient medium, it was necessary to determine their optimal ratio. For this purpose, testing of 25 experimental nutrient media with different amounts of these components was conducted. *M. bovis* accumulation was determined by titration and expressed in lg CFU/mL.

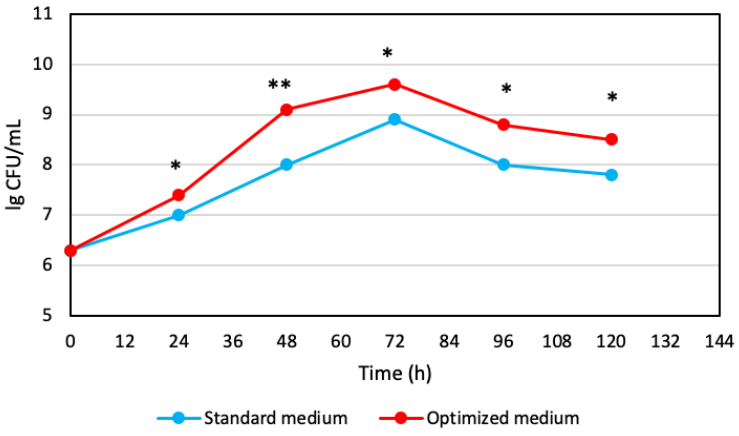
According to Table 2, the *M. bovis* maximum activity (9.60 lg CFU/mL) is observed when cultivated in a nutrient medium containing 12.5% fresh yeast extract and 25% horse serum.

The obtained results are confirmed by the data on changes in the biological activity of *M. bovis* "Kaluga 2020" isolate when cultured in a standard and optimized Hayflick nutrient medium (Fig. 5).

Table 2
Effect of nutrient medium composition on accumulation of *M. bovis* "Kaluga 2020" isolate

Experiment number	Contents of the studied components		lg CFU/mL
	fresh yeast extract, %	horse serum, %	
1	5.0	10	7.80
2	7.5	10	7.90
3	10.0	10	8.00
4	12.5	10	8.20
5	15.0	10	7.90
6	5.0	15	7.70
7	7.5	15	7.90
8	10.0	15	8.00
9	12.5	15	8.20
10	15.0	15	7.80
11	5.0	20	7.70
12	7.5	20	7.90
13	10.0	20	8.10
14	12.5	20	8.00
15	15.0	20	7.80
16	5.0	25	8.10
17	7.5	25	7.90
18	10.0	25	8.00
19	**12.5**	**25**	**9.60**
20	15.0	25	7.79
21	5.0	30	7.50
22	7.5	30	7.85
23	10.0	30	8.00
24	12.5	30	7.80
25	15.0	30	7.60

When cultured in an optimized medium the biological activity of *M. bovis* was on average 3.98×10^9 CFU/mL, which is 5 times higher than that of *M. bovis* when cultured in a standard Hayflick nutrient medium (0.79×10^9 CFU/mL).



** p < 0.05; ** p < 0.001*

Fig. 5. Activity of M. bovis "Kaluga 2020" isolate when cultivated in standard and optimized Hayflick medium

DISCUSSION

Based on the results of the conducted studies, it can be concluded that there is a correlation between the growth phase, the accumulation of mycoplasmas, the CFU indicator and the nutrient medium color (CCU parameter).

The transition from red to orange corresponded to the phase of exponential growth of mycoplasmas, and when the growth peak was reached, a change in the color of the medium from red-orange to orange was observed. During further cultivation the medium colour changed successively to orange-yellow and yellow, which corresponded to the decline phase as a result of cell lysis and intracellular ATP depletion. Similar results are confirmed by the foreign colleagues' data [15, 20].

Mycoplasma cultivation is considered a laborious technique [18], and the search for optimal media for obtaining high-quality biological material of these microorganisms, as well as for production of specific means of mycoplasmosis immunoprophylaxis is of urgent importance [21].

It was established that glucose had a minor effect on *M. bovis* growth and accumulation, which is consistent with the literature data [22, 23].

The use of optimized nutrient medium based on modified Hayflick broth containing 12.5% fresh yeast extract and 25% horse serum showed a 5-fold increase in accumulation of *M. bovis* "Kaluga 2020" isolate (3.98×10^9 CFU/mL) as compared to the standard medium (0.79×10^9 CFU/mL). The results obtained in similar studies on optimization of the nutrient medium composition for *M. hyopneumoniae* cultivation indicate only a 3-fold increase in mycoplasma accumulation as compared to the standard medium [13, 24].

Studying *M. bovis* growth, as well as determining the log phase, can play an important role in future investigations on isolation of this species mycoplasmas, cultivation and vaccine development.

CONCLUSION

The study results showed that after first 24 hours of cultivation in a liquid medium *M. bovis* "Kaluga 2020" isolate entered the phase of logarithmic growth and reached the maximum accumulation level in 72 hours. A decline phase occurred and the biological activity of the resulting material decreased with longer cultivation in 84 hours.

It was found that the growth factors – fresh yeast extract and horse serum – had the greatest effect on accumulation of *M. bovis* "Kaluga 2020" isolate when cultivated in Hayflick medium. The optimal content of these components in the culture medium was selected – 12.5 and 25%, respectively. An optimized nutrient medium based on modified Hayflick broth can be used to obtain bacterial material for the development of diagnostic products and means of specific prevention of *M. bovis*-induced diseases.

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INFORMATION ABOUT THE AUTHORS / ИНФОРМАЦИЯ ОБ АВТОРАХ

Mohammad Abed Alhussen, Post-Graduate Student, Department of Veterinary Medicine, Agrarian and Technological Institute, RUDN University, Moscow, Russia.

Alexander A. Nesterov, Candidate of Science (Veterinary Medicine), Senior Researcher, Reference Laboratory for Bovine Diseases, FGBI "ARRIAH", Vladimir, Russia.

Alexander V. Sprygin, Candidate of Science (Biology), Senior Researcher, Reference Laboratory for Bovine Diseases, FGBI "ARRIAH", Vladimir, Russia.

Irina N. Shumilova, Candidate of Science (Veterinary Medicine), Senior Researcher, Reference Laboratory for Bovine Diseases, FGBI "ARRIAH", Vladimir, Russia.

Maria S. Bryantseva, Leading Biologist, Laboratory for Porcine and Horned Livestock Prevention, FGBI "ARRIAH", Vladimir, Russia.

Olga P. Byadovskaya, Candidate of Science (Biology), Head of Reference Laboratory for Bovine Diseases, FGBI "ARRIAH", Vladimir, Russia.

Абед Алхуссен Мохаммад, аспирант департамента ветеринарной медицины аграрно-технологического института ФГАОУ ВО «Российский университет дружбы народов», г. Москва, Россия.

Нестеров Александр Александрович, кандидат ветеринарных наук, старший научный сотрудник референтной лаборатории болезней крупного рогатого скота ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Спрыгин Александр Владимирович, кандидат биологических наук, старший научный сотрудник референтной лаборатории болезней крупного рогатого скота ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Шумилова Ирина Николаевна, кандидат ветеринарных наук, старший научный сотрудник референтной лаборатории болезней крупного рогатого скота ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Брянцева Мария Сергеевна, ведущий биолог лаборатории профилактики болезней свиней и рогатого скота ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Бьядовская Ольга Петровна, кандидат биологических наук, заведующий референтной лабораторией болезней крупного рогатого скота ФГБУ «ВНИИЗЖ», г. Владимир, Россия.