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Molecular genetic and bacteriological methods of bovine mycoplasmosis diagnosis

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SUMMARY

Mycoplasmas are bacteria that are extremely unstable *in vitro* as they lack a rigid cell wall. They are most often detected in association with other pathogens, including those that can become L-forms if treated with antibiotics. *Mycoplasma* colonies, as well as colonies of L-form bacteria, have a typical "fried egg" appearance, therefore it is necessary to differentiate them for the accurate diagnosis and choice of treatment. The paper presents data on mycoplasma infection diagnosis in cattle and results of differentiation of isolated mycoplasma and L-form bacteria colonies using multiple passaging and real-time polymerase chain reaction. For that, 177 samples were collected from animals with mycoplasmosis clinical signs, 45 of them were tested using molecular genetic method, 132 samples were subjected to bacteriological testing. *Mycoplasma* DNA was detected in 71.1% of samples, and specific colonies were detected in 3.8% of samples. Such biochemical tests of mycoplasma species identification as arginine hydrolysis, blood serum liquefaction, film and grain formation, inoculation into Tween-80-containing medium, hemadsorption and hemolysis of erythrocytes do not allow an objective assessment of the species belonging to mycoplasmas, but, according to the results obtained, the isolated species most likely belongs to *Mycoplasma dispar*, which is pathogenic for cattle. Real-time polymerase chain reaction is undoubtedly the most accurate and rapid diagnostic method for mycoplasmosis, but a preliminary diagnosis can also be established bacteriologically within 2–7 days. In addition, during microbiological testing, it is possible to assess the antibiotic resistance of mycoplasma isolates, thereby developing an optimal and high-quality scheme of the disease treatment and prevention.

Keywords: *Mycoplasma*, bacteriological diagnosis, factor infection, microflora, pneumonia, L-form bacteria, specific nutrient media, molecular genetic diagnosis

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Молекулярно-генетический и бактериологический методы диагностики микоплазмоза крупного рогатого скота

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РЕЗЮМЕ

Микоплазмы – бактерии, лишенные ригидной клеточной стенки, поэтому крайне неустойчивы *in vitro*. Чаще всего их выявляют в ассоциации с другими возбудителями, среди которых есть те, что способны образовывать L-форму под действием антибиотических препаратов. Микоплазменные колонии, так же как и колонии L-форм, имеют вид «яичницы-глазуньи», поэтому для точного диагноза и выбора направления лечения необходимо провести их дифференциацию. В статье приведены данные о диагностике микоплазменной инфекции у крупного рогатого скота и результаты дифференциации выделенных колоний микоплазм и колоний L-форм бактерий методом многократных пассажей и с помощью полимеразной цепной реакции в режиме реального времени. Для выполнения поставленных задач были отобраны 177 образцов от животных, имеющих клинические признаки микоплазмоза, из них 45 исследованы молекулярно-генетическим методом, 132 – бактериологическим. При этом ДНК микоплазмы была выявлена в 71,1% проб, специфичные колонии – в 3,8% образцов. Такие тесты биохимической идентификации микоплазм, как гидролиз аргинина, разжижение сыворотки крови, образование пленки и пятен, посев на среду с Твином-80, гемадсорбция и гемолиз эритроцитов, не дают объективную оценку видовой принадлежности микоплазм, но, согласно полученным результатам, изолированный вид с наибольшей вероятностью относится к *Mycoplasma dispar*, являющейся патогенной для крупного рогатого скота. Несомненно, полимеразная цепная реакция в режиме реального времени – наиболее точный и быстрый метод диагностики микоплазмоза, но предварительный диагноз можно установить и бактериологически в течение 2–7 сут. Кроме того, при проведении микробиологических тестов возможно провести оценку антибиотикорезистентности изолированных микоплазм, тем самым разработать оптимальную и качественную схему лечения и профилактики заболевания.

Ключевые слова: микоплазма, бактериологическая диагностика, факторная инфекция, микрофлора, пневмония, L-форма бактерии, специфичные питательные среды, молекулярно-генетическая диагностика

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INTRODUCTION

Mycoplasmas (mollicutes) are very small microorganisms capable of passing through bacterial filters and reducing on cell-free nutrient media. They lack a rigid cell wall and are surrounded only by a three-layer cytoplasmic membrane, therefore they have a pronounced polymorphism, and round, oval and filamentous formations are found in smears. *Mycoplasmas* are extremely unstable *in vitro*. Their cytoplasmic membrane consists of sterol lipids, which brings it closer to eukaryotes and distinguishes it from other prokaryotes [1–3]. Among *Mycoplasmas* there are species that cause pathological processes in animals, as well as those that are closely linked with the cells of the macroorganism and do not provoke infection.

Mycoplasma bovis, *Mycoplasma mycoides*, *Mycoplasma bovigenitalium*, *Mycoplasma bovoculi*, *Mycoplasma dispar* pose the greatest etiological significance in cattle pathology [4, 5]. In animals the mycoplasmosis agents cause pneumonia of various severity, arthritis, conjunctivitis, vulvovaginitis, endometritis, mastitis, balanoposthitis, abortions and infertility of cows and bulls [4, 6, 7]. Lobar lung atelectasis, numerous necrotic foci, combined with mycoplasmosis infections, massive adhesions in the pleural cavity and abscesses in the lung tissue are observed during necropsy [8, 9].

Mycoplasmosis is widely spread not only in foreign countries but in the Russian Federation as well. In most cases the disease is asymptomatic and chronic; acute clinical manifestations are noted while the overall resistance of the body decreases in the autumn-winter and spring periods, it is often diagnosed randomly. *Mycoplasmas* are characterized by long-term persistence in the body. It is known that up to 40% of cows in a herd can suffer from mycoplasmosis [7, 10], resulting in huge economic damage to the establishment. No vaccination against this infection is carried out in our country, preventive measures shall be aimed at improving the animal feeding and keeping conditions [11], monitoring the quality of semen and sanitation of cows during the dry season, antibiotic prophylaxis may also be implemented.

Mycoplasmosis is often diagnosed in association with bacterial and viral infections such as leptospirosis, listeriosis, pasteurellosis, diplococcosis, infectious rhinotracheitis, viral diarrhea, parainfluenza and others [12–15].

Laboratory diagnosis of mycoplasmosis includes microbiological and molecular biological identification

of the pathogen. In order to detect the carrier animal in the herd, ELISA is used for the antibody level detection [16–18].

Mycoplasmas have high demands as regards the nutrient media composition for cultivation conditions, they are sensitive to the nutrient medium pH level, the optimal pH level for growth is 7.8–8.2. Yeast extract, glucose and blood serum are added to the nutrient media as sources of energy and sterol. In addition, most *Mycoplasma* species grow slowly, their cultivation lasts several weeks [19–21].

Identification of specific colonies requires their differentiation from L-form bacteria. The problem of *Mycoplasma*-infections is closely associated with the study of L-form bacteria. *Streptobacillus moniliformis* species first discovered in the culture in 1935, were named the L-forms and, due to their extraordinary similarity to *Mycoplasmas* were originally assigned to the group of pleuropneumonia-like organisms (PPLO). The study of L-forms properties showed that some of them can revert to bacterial form, while others cannot, so they received the term "stable L-forms". The term *M. mycoides* was adopted for the bovine peripneumonia agent in 1956 [22].

The main aspects of studying biology of L-forms and the *Mycoplasma* family should include: the study of their morphological and physiological characteristics; the study of the mechanisms of transformation of bacteria into L-forms; the development of criteria for differentiating L-forms and *Mycoplasmas*, aimed at revealing the role of L-forms in the phylogeny of the *Mycoplasmataceae* family and serving as the basis for the most rational classification scheme of these forms; elucidation of the role of L-forms of bacteria and the *Mycoplasmataceae* family in pathological processes whose infectious nature does not fit into bacterial or viral etiology [23].

The morphological traits of L-forms and *Mycoplasmas*, namely the lack of a rigid cell wall, determine their high plasticity, fragility and pronounced polymorphism. The physiological traits of L-forms and *Mycoplasmas* are largely related to their thin structure. Both groups are similar in composition as regards proteins, carbohydrates and lipids, and lytic agents that destroy lipoproteins dissolve both groups of microorganisms [24]. Osmotic stress does not have a drastic effect on *Mycoplasmas* and salt-independent L-forms [25].

Mycoplasmas, as well as L-forms, are resistant to those antibiotics and drugs, the initial effect of which is associated with inhibition of the cell wall synthesis of microorganisms. Lysozyme, which is known to impact *β*-glucoside linkages of the cell membrane, does not impact either L-forms or *Mycoplasmas*.

Polymerase chain reaction (PCR) is an adequate, highly sensitive, modern diagnostic method for mycoplasmosis aimed at DNA detection and species identification of mycoplasmosis pathogens. This method is widely used in veterinary practice, mainly for mass tests in combination with other methods or as a rapid diagnosis method [26, 27].

The aim of this work was to study the cultural and morphological properties of *Mycoplasmas* and their differentiation from L-form bacteria, as well as to search for highly sensitive and qualitative methods of diagnosis and differential diagnosis of bovine mycoplasmosis.

MATERIALS AND METHODS

The study was carried out in the Vologda Branch of the FSC VIEV, the FSC VIEV Moscow and the SFVIVO "Vologda Oblast Laboratory".

The following served as the materials for the bacteriological study: cervical mucus ($n = 35$) and udder secretions of cows ($n = 30$), nasal ($n = 58$), conjunctival ($n = 6$) and prepucial mucus ($n = 1$) of calves of different age, as well as pathologic and anatomical material from two calves – samples of lung tissue (a section of relatively healthy tissue, a section on the margin of pathology and healthy tissue, a section with pathological lesions) and samples of mediastinal lymph nodes with undamaged morphological structure.

Cervical ($n = 18$), nasal ($n = 18$), conjunctival ($n = 2$), prepucial mucus ($n = 1$), blood ($n = 1$) and pathologic-anatomical material ($n = 2$) from calves of different age were used for PCR testing.

The primary inoculation of the material was carried out in liquid and solid nutrient media prepared on the basis of meat-peptone broth or agar and Martin broth, with the addition of 20% horse blood serum, 10% yeast extract and extraneous microflora growth inhibitors. Subsequent inoculations were carried out on a solid nutrient medium, using the method of agar blocks, as well as homogenization of agar blocks in saline solution. When specific colonies were detected, they were microscopically studied and identified. Growth on a liquid nutrient medium was daily monitored, the degree of turbidity, the presence of sediment and film were visually assessed for 14 days. Then the material was inoculated onto a solid nutrient medium and observed for 14 days [3, 20].

The primers to detect *Mycoplasma* DNA were used in real-time PCR in accordance with the instructions to the "PCR-MYCOPLASMOSIS-FAKTOR" reagent kit (OOO "VET FAKTOR", Russia).

The tests were conducted according to "Guidelines for isolation, cultivation and identification of mycoplasmas, achholeplasmas and ureaplasmas" [20].

The study of some cultural and enzymatic signs, including film and stain formation, inoculation into Tween-80-containing medium (for typing as *M. bovis*), arginine hydrolysis, blood serum liquefaction, hemadsorption and hemolysis of colonies, – was carried out according to the methods of L. Shtipkovich [1].

RESULTS AND DISCUSSION

Biomaterial samples were collected from animals with clinical manifestations of mycoplasmosis: chronic mastitis, endometritis, a history of abortions in cows; rhinitis, conjunctivitis, balanoposthitis, arthritis and pneumonia in calves. The samples were kept in saline solution before being sent to the laboratory.

A total of 45 samples were collected for real-time PCR testing. The results are presented in the table.

It was found that 32 (71%) out of 45 samples were positive. The *Mycoplasma* DNA was detected in the cervical mucus of the mother and the nasal mucus of the calf in one case; the *Mycoplasma* DNA was detected in nasal mucus and was not detected in the blood of calves in five cases.

One hundred and thirty-two biological samples were bacteriologically tested. The specific *Mycoplasma* colonies

Table
Real-time PCR results for samples tested for presence of *Mycoplasma* spp. DNA

Tested material	Detection of *Mycoplasma* spp. DNA		
	DNA detected	DNA not detected	Total
Cervical mucus (cows)	11	7	18
Nasal mucus (calves)	17	1	18
Conjunctival mucus (calves)	2	0	2
Blood (calves)	0	5	5
Pathological material	2	0	2
Total	32	13	45

were isolated in only five samples (3.8%), including four nasal mucus samples and one sample of pathological material (mediastinal lymph node tissue) from a dead calf.

Primary inoculation of anatomical pathology samples of lung tissue and mediastinum lymph nodes was carried out onto a liquid nutrient medium. No broth turbidity, film or sediment formation were recorded during 14 days of observation. After inoculation onto a solid nutrient medium within the following 14 days, the colonies morphologically similar to *Mycoplasma* spp. colonies were detected.

As it can be seen in Figure 1, the colonies have a typical "fried egg" appearance with a raised center and a lighter in colour peripheral part.

The figure also demonstrates growth of extraneous microflora, so there were doubts whether the colonies belonged to *Mycoplasma* species. As a result of staining smears of nonspecific colonies according to Gram and Romanowsky – Giemsa, the bacteria similar in structure to unicellular fungi (identification was not carried out) were detected.

Subsequent tests were aimed at differentiating between *Mycoplasma* colonies and L-form bacteria colonies and obtaining a pure culture of these *Mycoplasma* species by multiple passaging.

The needed colonies were excised point-by-point under a microscope and inoculated into solid and liquid nutrient media with and without inhibitors, and the blocks were suspended in saline. The growth of *Mycoplasmas* was observed at day 1–3 (Fig. 2).

The test showed that the colonies remained similar to *Mycoplasma* ones, but already had a more pronounced central part. To confirm the test result, the colonies were placed in a saline solution and a PCR test for belonging to *Mycoplasma* spp. was performed. All samples were positive.

The cervical, nasal, conjunctival, prepucial mucus and breast secretions were initially inoculated onto a solid nutrient medium. At the same time, specific colonies in nasal mucus inoculation were detected after 48 hours. They were also assigned to *Mycoplasma* spp. based on the results of the molecular genetic method and multiple passaging.

Some biochemical tests have shown that isolated strains most likely belong to *M. dispar*, which is the causative agent of bovine mycoplasmosis.

Figure 3*a* shows *Mycoplasma* growth in a Tween-80-containing medium, which is used for the test for typing as belonging to *M. bovis*. The test result is considered positive if a light ring forms around the colonies, a negative result can be seen in the photo.

The redness of the medium (increased pH) indicates the arginine hydrolysis by *Mycoplasma* (Fig. 3*b*). It should be noted that *M. alcalescens*, *M. arginini*, *M. canadense* possess this biological property.

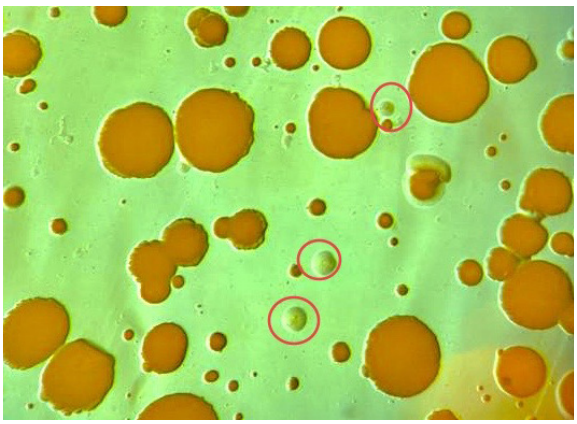

Fig. 1. Growth of mixed microflora in solid enriched nutrient medium, Mycoplasma colonies are circled in red (MBS-10 stereoscopic microscope, 14 × 2 magnification)

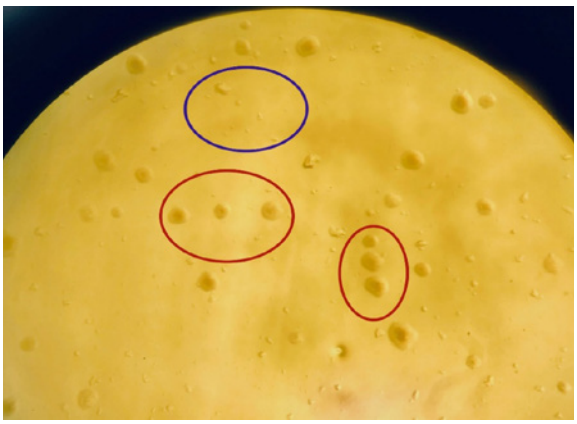

Fig. 2. Pure Mycoplasma culture: Mycoplasma colonies are circled in red, parts of nutrient medium from the previous passage are circled in blue (MBS-10 stereoscopic microscope, 14 × 2 magnification)

The serum liquefaction test used for identification of *Mycoplasma* had a positive result: a liquid was formed, cavities and ruptures appeared (Fig. 3*c*).

It should be also mentioned that no formation of films (cholesterol and phospholipids) and grains (precipitated magnesium and calcium salts) was noted during growth in nutrient media, which is indicative of absence of *Mycoplasma* lipolytic action. A negative result was obtained when hemadsorbent and hemolytic properties were studied using bovine red blood cells.

CONCLUSION

Based on the presented results, the fastest and most accurate diagnosis of bovine *Mycoplasma* infection includes conducting molecular genetic tests. However, a preliminary diagnosis can also be established bacteriologically within 2–7 days. In addition, microbiological tests allow assessing the antibiotic resistance of isolated *Mycoplasma*, thereby developing an optimal and high-quality treatment and prevention scheme. False-negative results might be obtained when using molecular genetic and bacteriological methods of mycoplasmosis diagnosis, but these results may occur more often when the bacteriological method is implemented due to the low growth rate of *Mycoplasma* and the resistance of the associated microflora to antimicrobial agents included in nutrient media composition.

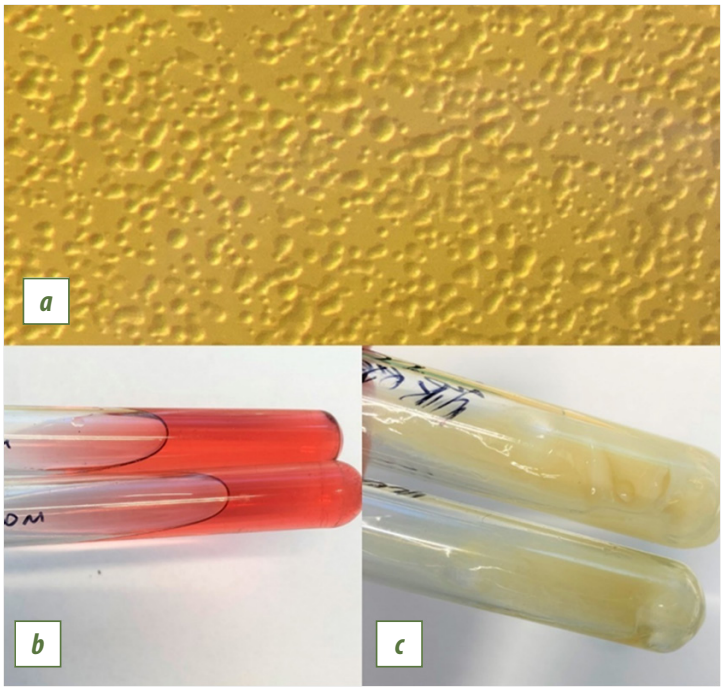

Fig. 3. Biochemical properties of isolated Mycoplasma: a – inoculation into medium supplemented with Tween-80 (test for typing as M. bovis); b – hydrolysis of arginine; c – liquefaction of blood serum

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