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Validation and application of qPCR test kit for detection of *Mycoplasma dispar* DNA

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ABSTRACT

Introduction. Currently, *Mycoplasma dispar* is widely spread and circulating in livestock farms around the world, including in the Russian Federation. The implementation of a real-time polymerase chain reaction test kit for detecting *Mycoplasma dispar* DNA in veterinary practice is highly relevant, as this pathogen can cause respiratory diseases in cattle and contribute to calf mortality, leading to significant economic losses in livestock production.

Objective. To introduce a newly developed real-time polymerase chain reaction test kit *Mycoplasma dispar* DNA detection kit into veterinary practice and determine its major validation parameters.

Materials and methods. *Mycoplasma dispar* reference strain (ATCC No. 27140) was cultured in 1699 Revised Mycoplasma Medium recommended by the American Type Culture Collection. DNA was extracted using a commercial kit, real-time polymerase chain reaction was performed using pre-selected parameters. The major validation parameters of the test kit were determined: analytical sensitivity, analytical specificity, amplification efficiency, repeatability and reproducibility. Applicability of real-time polymerase chain reaction test kit for detection of *Mycoplasma dispar* DNA was demonstrated.

Results. The *Mycoplasma dispar* DNA detection test kit demonstrated an analytical sensitivity (detection limit) of 10 copies/μL (100 copies/reaction), 100% specificity (exclusive to *Mycoplasma dispar* DNA), 99.01% amplification efficiency, and an average repeatability coefficient of variation of 0.91%. Reproducibility coefficient of variation ranged from 0.66% to 1.26% across 5 replicates and was 0.91% across 15 replicates. The test kit was validated using 228 biological samples from cattle from 13 regions of the Russian Federation, while *Mycoplasma dispar* DNA was detected in 39.47% of the samples tested.

Conclusion. The developed *Mycoplasma dispar* DNA test kit has demonstrated high validation performance and is suitable for diagnosing bovine mycoplasmosis.

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Валидация и применение тест-системы на основе метода ПЦР в режиме реального времени для выявления ДНК *Mycoplasma dispar*

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РЕЗЮМЕ

Введение. В настоящее время наблюдается широкое распространение и активная циркуляция *Mycoplasma dispar* в животноводческих хозяйствах по всему миру, в том числе и в Российской Федерации. Актуальность внедрения в ветеринарную практику тест-системы на основе полимеразной цепной реакции в режиме реального времени для выявления ДНК *Mycoplasma dispar* обусловлена важной ролью данного возбудителя в развитии комплекса респираторных болезней крупного рогатого скота, приводящих к значительным экономическим потерям в животноводстве, в том числе являющихся причиной падежа телят.

Цель исследования. Определение основных валидационных характеристик и внедрение в ветеринарную практику разработанной тест-системы для выявления ДНК *Mycoplasma dispar* методом полимеразной цепной реакции в режиме реального времени.

Материалы и методы. Референтный штамм *Mycoplasma dispar* (ATCC № 27140) культивировали на питательной среде 1699 Revised Mycoplasma Medium, рекомендованной American Type Culture Collection. ДНК выделяли с использованием коммерческого набора, постановка ПЦР в режиме реального времени осуществлялась по заранее подобранным параметрам. Были определены основные валидационные характеристики тест-системы: аналитическая чувствительность, аналитическая специфичность, эффективность амплификации, повторяемость и воспроизводимость. Показана возможность практического применения тест-системы на основе ПЦР в режиме реального времени для выявления ДНК *Mycoplasma dispar.*

Результаты. Аналитическая чувствительность (предел обнаружения) тест-системы по выявлению ДНК *Mycoplasma dispar* составила 10 копий ДНК/мкл (или 100 копий ДНК/реакцию), специфичность – 100% (детектирует только ДНК *Mycoplasma dispar*), эффективность амплификации – 99,01%, среднее значение коэффициента вариации при оценке повторяемости – 0,91%, воспроизводимости – 0,66–1,26% в рамках 5 повторений и 0,91% в рамках 15 повторений. Апробацию тест-системы проводили на 228 пробах биоматериала, отобранных от крупного рогатого скота из 13 регионов Российской Федерации, при этом ДНК *Mycoplasma dispar* была обнаружена в 39,47% исследуемых проб.

Заключение. Разработанная тест-система для выявления ДНК *Mycoplasma dispar* продемонстрировала высокие валидационные показатели и может быть использована в диагностике микоплазмоза крупного рогатого скота.

Ключевые слова: *Mycoplasma dispar,* тест-система, ПЦР-РВ, внутренний контрольный образец, чувствительность, специфичность, эффективность амплификации

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INTRODUCTION

Mycoplasma dispar is a globally prevalent pathogen responsible for respiratory diseases in cattle, particularly in calves. *M. dispar* infection typically manifests as mucopurulent inflammation of the upper respiratory tract with frequent lung involvement, resulting in substantial economic losses in cattle production [1, 2, 3].

Mycoplasma dispar, a member of the genus *Mycoplasma* (class *Mollicutes*), lacks a cell wall, is pleomorphic, and has a compact 803 kb genome with a low G + C composition (28.5–29.3 mol%) [4, 5].

Mycoplasma dispar requires a specific medium to enable growth and its colonies do not show – especially during the early passages – the typical "fried-egg" appearance. In addition, *M. dispar* are not always inhibited by hyperimmune serum, making conventional identification difficult in the early stages [5]. *M. dispar* glucose fermentation aligns with its phylogenetic cluster (hominis group), but unlike some mycoplasmas, it lacks arginine hydrolysis. Tetrazolium reduction occurs under both oxygen conditions. The bacterium has no phosphatase activity [6, 7, 8]. *M. dispar* produces an outer capsule consisting of a polysaccharide identified as a polymer of galacturonic acid [9]. The capsule appears to be produced during infection in response to mammalian host cells, and may exert an inhibitory effect on the activity of bovine alveolar macrophages and prevent their activation [10].

It has been reported that mycoplasma can be inhibited by alveolar macrophages if anti-capsular polysaccharide antibodies are present [9].

Mycoplasma dispar was first isolated from the lungs of pneumonic calves in England in 1969. Then these mycoplasmas were reported from Denmark, Belgium, the Netherlands, France, Australia, the USA, Canada, Korea and Japan [8]. In Europe, *M. dispar* infection was reported from the UK, and recently from Brazil and Italy [11, 12, 13, 14, 15]. In the Russian Federation, testing of 1,186 biological samples collected from cattle with respiratory and/or reproductive diseases from 34 different regions during 2015–2018 revealed *M. dispar* DNA in 37.15% of samples by agarose gel electrophoresis of polymerase chain reaction (PCR) products [16].

Mycoplasma dispar is transmitted between animals through respiratory secretions and can be detected in the respiratory tracts of both healthy calves and those with pneumonia [3]. Mycoplasma infections, particularly when combined with other respiratory pathogens under suboptimal conditions, represent a significant threat to animal health and productivity in high-density livestock operations [17, 18]. In the Netherlands, *M. dispar* was isolated from 92% of pneumonic calves and from only 40% of healthy calves [7, 19]. In Denmark *M. dispar* was found in over half of calf lungs showing either fibrino-necrotising or suppurative bronchopneumonia [20]. Other bacterial pathogens, including *Histophilus somni, Pasteurella multocida, Arcanobacterium pyogenes,* and *Mannheimia haemolytica*, were co-detected with mycoplasmas. *M. dispar* is detected frequently from pneumonic calves in the UK, and is believed to be the cause of a severe pleuropneumonia similar to the WOAH (World Organization for Animal Health) listed contagious bovine pleuropneumonia [21].

Few detailed studies have been carried out to establish the exact role of *M. dispar* in bovine respiratory disease (BRD) which is a chronic condition affecting beef cattle in feedlots and dairy calves. It is estimated to cost the USA cattle industry alone over US $4 billion in production losses, treatment and prevention [21].

Calves affected by BRD are usually treated with antimicrobials even though some of these are not effective against mycoplasmas [22]. Consequently, there is a real danger of the emergence of resistance as a result of chronic therapies with multiple antimicrobials, which is already observed for the other BRD bacterial pathogens [23].

The pathogenicity mechanisms have been identified in *M. dispar* and include its ability to produce hydrogen peroxide and biofilm, both well-known virulence factors.

Studies have shown that *M. dispar* is able to colonize the epithelial cells of the respiratory tract exerting cytostatic and even cytopathic effects on bronchial and bronchiole cells, potentially impairing the clearance of bacteria [9, 21, 24]. Furthermore, *M. dispar* has been shown to be immunosuppressive in the host [25]. For these reasons *M. dispar* is included with *M. bovis* amongst the agents that cause or exacerbate BRD [8, 21]. *M. dispar* is causally associated with exudative bronchitis and interstitial pneumonia in calves, with transmission occurring primarily via airborne respiratory droplets and sustained close contact [26].

Mycoplasma dispar causes purple to red consolidation mainly in the cranioventral areas of the lung [27]. *M. dispar* were shown to be capable of causing a mild sub-clinical bronchiolitis with lymphoid cuffing in gnotobiotic calves [10]; occasional cases of mastitis were also reported [28]. Other authors report that *M. dispar* is frequently related to alveolitis, in which neutrophils, macrophages, and edema liquid aggregate in the alveolar wall and spaces [26, 29]. Field cases of subclinical pneumonia from which *M. dispar* was isolated had similar lesions [30].

The genomic information of *M. dispar* in publicly available databases is limited. The genome succession of *M. dispar* reference strain ATCC 27140 was delivered in the GenBank in 2015. Based on the phylogenetic analysis of 16S rRNA gene sequence *M. dispar* was clustered with *M. ovipneumoniae, M. flocculare* and *M. hyopneumoniae,* isolated from sheep and goats [31].

Controlling mycoplasmosis effectively requires a multi-faceted approach that includes minimizing environmental stress, ensuring proper animal husbandry, and maintaining good air circulation within facilities. Measures to prevent infection of calves from adult animals are required [8].

Timely diagnosis is one of the key components in bovine mycoplasmosis control. Laboratory confirmation of *M. dispar* infection is clinically important, as this globally distributed pathogen significantly impacts livestock health and production [18, 32, 33, 34].

Conventional identifying bovine mycoplasmas through isolation on nutrient media is a crucial first step, as it allows for detailed examination of their cultural, morphological, and biological characteristics. This method also enables the establishment of a clinical isolate bank, which can support future development of improved prevention, control, and eradication strategies for mycoplasmosis, as well as facilitate antimicrobial resistance monitoring [8, 18, 33, 34]. However, it should also be noted that this method is laborious and requires 7–10 days for diagnosis [3, 18, 34].

While not widely used, serological tests like radial hemolysis, ELISA [35], and passive hemagglutination have been reported for detecting *M. dispar* antibodies [36, 37]. The authors described that weak antibody presence against *M. dispar* in cattle, despite its surface-lung location, might be due to the relatively low sensitivity of the serological tests used, rather than the animal's immune system failing to mount an adequate response [35].

While PCR has significantly improved mycoplasma detection, it was not until 2004 that a PCR method specific to the *M. dispar* genome was reported [38]. Both specific and universal oligonucleotides were utilized to detect single nucleotide polymorphisms in the 16S rRNA gene sequence. The PCR/DGGE technique (denaturing gradient gel electrophoresis) was also described, which can detect and identify more than 70 different mycoplasmas, including *M. dispar* [39, 40].

Contemporary diagnostics can achieve high efficiency through real-time PCR (qPCR), enabling rapid and precise detection of specific mycoplasma genomic loci in biological samples [18, 32, 34, 41].

The use of fluorescently labeled TaqMan probes that bind to a specific sequence within the amplified DNA region (the interprimer segment) significantly enhances the specificity of PCR-based assays. Real-time PCR, unlike traditional PCR, doesn't require post-amplification handling of the sample, reducing the risk of contamination and leading to faster, more efficient analysis.

The incorporation of an internal control sample (ICS) in commercial qPCR assays ensures result reliability by detecting PCR inhibition, thereby preventing false-negative interpretations [42]. An important need in qPCR test kit development is incorporating ICSs to monitor both nucleic acid extraction and the subsequent amplification process [43, 44, 45, 46].

It is also worth noting that, in accordance with the WOAH requirements, it is recommended to include ICS into each PCR test for quality control [47].

Tools for the molecular identification of mycoplasma genomes, including of *M. dispar*, enable not only to monitor the animal disease situation in domestic farms, but also to control both the import of animals and their use for production purposes [34, 48].

Currently, there are no commercially available domestic qPCR test kits to detect *M. dispar* DNA in the Russian Federation, and therefore the development of a specific qPCR diagnostic test kit and its introduction into veterinary practice is an urgent task [34].

The purpose of this work was to determine the main validation characteristics of the qPCR test kit for detection of *M. dispar* DNA developed by the Federal Centre for Animal Health, and to introduce it into veterinary practice.

MATERIALS AND METHODS

Bacteria and viruses. The *M. dispar* reference strain (ATCC No. 27140), delivered from the collection of strains of microorganisms of the Federal Centre for Animal Health, and biological samples collected from cattle of various age groups (stabilized blood; nasal and tracheal swabs; pieces of lungs, trachea and lymph nodes; pleural fluid) were used.

To assess the analytical specificity of the developed test kit, the following bacterial strains were used: *M. bovis* ATCC No. 25523, *M. bovigenitalium* ATCC No. 19852, *M. bovis* Kaluga 2020, *Mycoplasma mycoides* subsp. *mycoides* SC (MmmSC) Madugri-8 DNA (Federal Research Center of Virology and Microbiology, Russia), *Mycoplasma mycoides* subsp. *mycoides* SC (MmmSC) "T1/44/ARRIAH", and other bacterial and viral agents responsible for analogous diseases in cattle: *Escherichia coli* "EC-21", *Mannheimia haemolytica* "No. 1412", *Pasteurella multocida* " No. 1414", *M. bovigenitalium* isolate, *M. dispar* isolate, *M. bovis* isolate, bovine parainfluenza 3 virus "VGNKI-4", bovine respiratory syncytial virus "Vologda/2020", bovine viral diarrhea virus "NADL-ARRIAH".

Mycoplasma dispar culture. The *M. dispar* reference strain (ATCC No. 27140) was cultured on the 1699 Revised Mycoplasma Medium nutrient medium recommended by the American Type Culture Collection, which contained 7.5 g of brain

Table 1
Threshold cycle (Ct) values of optimized real-time polymerase chain reaction temperature-time profile for *M. dispar* DNA detection (*n* = 3)

Stage	Temperature	Duration	Number of cycles	Mean value Ct ± SD
Profile 1				
Heating of the reaction mixture	95 °C	5 minutes	1	25.46 ± 0.37
Denaturation	95 °C	15 seconds	40	
Primer annealing and elongation	60 °C	60 seconds (fluorescence measurement of Green/FAM, Red/Cy5)		
Profile 2				
Heating of the reaction mixture	95 °C	5 minutes	1	26.30 ± 0.86
Denaturation	95 °C	10 seconds	40	
Primer annealing	60 °C	20 seconds (fluorescence measurement of Green/FAM, Red/Cy5)		
Elongation	72 °C	20 seconds		
Profile 3				
Heating of the reaction mixture	95 °C	5 minutes	1	28.87 ± 0.15
Denaturation	95 °C	10 seconds	45	
Primer annealing	58 °C	20 seconds (fluorescence measurement of Green/FAM, Red/Cy5)		
Elongation	72 °C	20 seconds		

heart infusion broth; 40.0 µL of 10× Hank's Balanced Salts Solution; 10.0 µL of 0.25% phenol red solution; 200.0 µL of heat inactivated porcine serum; 100.0 µL of 5% lactalbumin hydrolysate in 1× phosphate buffer solution; 20.0 µL of yeast extract and 660.0 µL of distilled water. The bacteria were cultured in an incubator at (37 ± 0.5) °C and 5% CO_2 for 5 days. After incubation, turbidity in test tubes and discoloration of the medium (yellowing) were observed, *M. dispar*-like colonies formed on a solid nutrient medium; *M. dispar* biological activity was determined by colony-forming unit counting (CFU/µL) [49, 50].

DNA was extracted using a commercial Ampli Prime Ribosorb kit (Central Research Institute of Epidemiology, Rospotrebnadzor, Russia) in accordance with the manufacturer's instruction.

Real-time PCR protocol. The reaction mixture for amplification per one reaction contained the following components: PCR-buffer-B for Taq DNA polymerase 10× (Syntol, Russia); 5 U/µL of SynTaq DNA polymerase with enzyme-inhibiting antibodies (Syntol, Russia); 25 mM of aqueous solution of magnesium chloride $MgCl_2$ (Syntol, Russia); 100 mM of aqueous solutions of four deoxynucleoside triphosphates (dNTP): dATP, dGTP, dTTP, dCTP (a common mixture of dNTP is prepared and diluted with nuclease-free water to a concentration of 10 mM of each dNTP; Fermentas, Lithuania); direct primer (100 pmol/µL), reverse primer (100 pmol/µL) and a TaqMan probe (100 pmol/µL) to detect a region of the gene encoding *M. dispar*16S rRNA (Syntol, Russia); direct primer (100 pmol/µL), reverse primer (100 pmol/µL) and TaqMan probe (100 pmol/µL) for detection of artificially synthesized ICS (Syntol, Russia) [51]. The resulting volume of the reaction mixture was adjusted to 15 µL with deionized, nuclease-free water (Eurogen, Russia). After that, 15 µL of the reaction mixture and 10 µL of the DNA matrix of the tested samples were added to the prepared tubes. Amplification was performed in a Rotor-Gene real-time PCR cycler (QIAGEN, Germany).

A plasmid construct containing the oligonucleotide sequence of the genome region (with an initial concentration of 2×10^7 copies/µL) and *M. dispar* genome specific region (target fragment) was used as a positive control sample.

Deionized, nuclease-free water (Eurogen, Russia) was used as a negative control sample (NCS) and as a negative PCR control.

Validation. The validation parameters of the test kit were determined according to the recommended method by S. A. Bustin et al. guidelines for publication of developed quantitative PCR protocols [52].

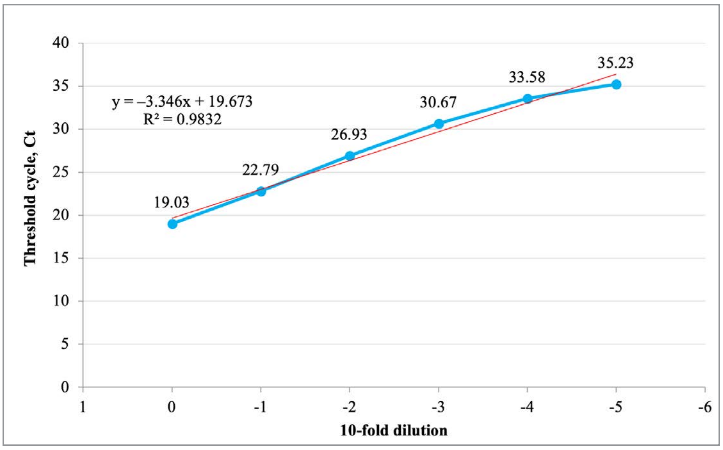

Fig. 1. Linear correlation of real-time polymerase chain reaction results for 10-fold dilutions of the M. dispar genome DNA

Table 2
Specificity assessment of the developed real-time polymerase chain reaction kit for *M. dispar* DNA detection ($n = 3$)

Genetic material	Strain	qPCR result, Green/FAM (*M. dispar* DNA)	qPCR result, Red/Cy5 (ICS DNA)
Escherichia coli	"EC-21"	neg.	+
Mannheimia haemolytica	"No. 1412"	neg.	+
Pasteurella multocida	"No. 1414"	neg.	+
Mycoplasma bovigenitalium	ATCC No. 19852	neg.	+
Mycoplasma bovigenitalium	isolate	neg.	+
Mycoplasma dispar	ATCC No. 27140	pos.	+
Mycoplasma dispar	isolate	pos.	+
Mycoplasma bovis	ATCC No. 25523	neg.	+
Mycoplasma bovis	"Kaluga 2020"	neg.	+
Mycoplasma bovis	isolate	neg.	+
Mycoplasma mycoides subsp. *mycoides* SC (MmmSC)	"T1/44/ARRIAH"	neg.	+
Mycoplasma mycoides subsp. *mycoides* SC (MmmSC)	Madugri-8	neg.	+
Bovine parainfluenza virus-3	"VGNKI-4"	neg.	+
Bovine respiratory syncytial virus	"Vologda/2020"	neg.	+
Bovine viral diarrhea virus	"NADL-ARRIAH"	neg.	+
Nuclease-free water	–	neg.	+

neg. – *M. dispar* DNA was not detected; pos. – *M. dispar* DNA was detected;
"+" – ICS DNA was detected.

Table 3
Variability of real-time polymerase chain reaction Ct values for *M. dispar* ($n = 15$)

Run	Replicate	Ct value	Mean Ct value	Standard deviation (SD)	Coefficient of variation (Cv, %)
I	1	27.09	26.74	0.22	0.82
	2	26.76			
	3	26.51			
	4	26.68			
	5	26.64			
II	1	27.41	27.36	0.18	0.66
	2	27.50			
	3	27.54			
	4	27.13			
	5	27.20			
III	1	26.58	27.00	0.34	1.26
	2	26.93			
	3	27.32			
	4	27.38			
	5	26.77			
		Total	27.03	0.25	0.91

To assess the specificity of the developed qPCR, biological samples containing *Mycoplasma* DNA and viral and bacterial nucleic acids which can cause similar diseases in cattle were tested. The test kit sensitivity was assessed using known positive biological samples containing *M. dispar* DNA.

The limit of *M. dispar* DNA detection (analytical sensitivity) of the developed test kit was assessed using a positive control sample containing *M. dispar* DNA with an initial concentration of 2×10^7 copies/µL, each dilution was tested in 5 replicates.

PCR amplification efficiency was assessed using serial 10-fold dilutions of a positive sample of biological material containing *M. dispar* DNA in 3 replicates and calculated according to the formula:

$$E = (10^{1/\text{slope}} - 1) \times 100\%,$$

where slope is standard curve (plot of Ct vs. $\log_{10}$ input template concentration).

The data were statistically analyzed using Microsoft Excel, including calculations of mean values with standard deviations (± SD), regression analysis, and coefficients of variation. The coefficient of variation (CV) for reproducibility and repeatability should not exceed 10%.

Intermediate precision (reproducibility) was assessed by testing the same sample in 5 replicates in 3 independent qPCR runs ($n = 15$ total replicates).

RESULTS AND DISCUSSION

The Federal Centre for Animal Health developed and validated Russia's first qPCR test kit for *Mycoplasma dispar* DNA detection, incorporating an ICS.

The use of ICS improve PCR reliability and accuracy, particularly by identifying reaction inhibition. The Laboratory Technology Committee of the American Association of Veterinary Laboratory Diagnosticians (AAVLD) has recommended to its membership that all new molecular assays being validated and implemented include an inhibition monitoring strategy based on internal validation for the host, target species, and sample matrix combination being tested [53].

The initial validation step involved identifying a temperature profile that optimizes both the sensitivity and amplification efficiency of the test kit.

The qPCR temperature-time profile was optimized using pre-established reaction component concentrations to achieve consistent threshold cycle (Ct) values. The results are given in Table 1.

The following qPCR temperature-time profile was considered optimal for *M. dispar* DNA detection: 5 minutes at 95 °C (heating of the reaction mixture), followed by 40 PCR cycles consisting of DNA denaturation for 15 seconds at 95 °C, primer annealing and cDNA elongation for 60 seconds at 60 °C.

Validation testing confirmed that the developed kit eliminates false-positive results when challenged with DNA from bovine respiratory disease-associated bacteria. It was shown that the test kit has 100% analytical specificity for *M. dispar* (Table 2), which is higher than demonstrated by J. B. W. J. Cornelissen et al., in which the specificity was 98.2% [54]. The oligonucleotides were verified using the NCBI BLAST database, confirming no significant homology with other *Mycoplasma* species and demonstrating specificity for the *M. dispar* genome.

The amplification efficiency was evaluated using serial 10-fold dilutions of a positive sample containing *M. dispar* DNA. Based on the obtained Ct values for each dilution, the amplification efficiency (E) was 99.01%, which was more significant than the 97.49% efficiency demonstrated by J. B. W. J. Cornelissen et al. during the development of a single PCR [54]. Linear correlation value (R2) was 0.9832 (Fig. 1).

In-laboratory precision under reproducibility conditions was evaluated by examining how consistently measurements are obtained when varying personnel, time, and equipment. The tests were performed using biological material containing and not containing *M. dispar* DNA. Recent studies have shown that the results of *M. dispar* DNA detection using the developed test kit are fully consistent with the expected results and do not depend on time, personnel, or equipment.

Intermediate precision under reproducibility (repeatability) conditions was assessed by testing the same sample in five replicates in three independent qPCR runs ($n = 15$ total replicates).

The threshold cycle (Ct) mean value in three PCR runs ranged from 26.74 to 27.36 with a standard deviation (SD) of 0.18 to 0.34. The coefficient of variation (CV) of 0.91% is well within the acceptable range of 10%. When summing up the results of three qPCR runs, the mean Ct value and the standard deviation was 27.03 and ± 0.25, respectively (Table 3).

At the next stage, the detection limit (analytical sensitivity) was determined for *M. dispar* DNA isolated from serial 10-fold dilutions of an artificially synthesized DNA sequence corresponding to a specific region of the *M. dispar* genome, starting at an initial concentration of 2×10^7 DNA copies/µL. Each dilution was tested in five replicates (Table 4).

It has been established that for the developed qPCR-based test kit, the detection limit of *M. dispar* DNA is 10 DNA copies/µL (100 DNA copies/reaction).

In studies conducted by L. McAuliffe et al., DGGE of the 16S ribosomal DNA PCR product was used to differentiate 32 mycoplasma species. This method is a valuable tool for quickly identifying various *Mycoplasma* species, especially when specific PCR tests are not available [39, 40]. However, artifacts introduced during PCR or the subsequent DGGE analysis lead to skewed results when this method is used for quantitative analysis of *α*-diversity or relative operational taxonomic unit abundance [55].

While some researchers have used conventional PCR to detect *M. dispar* [31, 38, 56], qPCR offers faster and more accurate results without the need for gel electrophoresis [57].

Table 4
Analytical sensitivity (detection limit) of the real-time polymerase chain reaction test kit for *M. dispar* DNA detection

Matrix	*M. dispar* DNA detection, replicate					Matching the expected result,%
	1	2	3	4	5	
2×10^7 DNA copies/µL	pos.	pos.	pos.	pos.	pos.	100
2×10^6 DNA copies/µL	pos.	pos.	pos.	pos.	pos.	100
2×10^5 DNA copies/µL	pos.	pos.	pos.	pos.	pos.	100
2×10^4 DNA copies/µL	pos.	pos.	pos.	pos.	pos.	100
2×10^3 DNA copies/µL	pos.	pos.	pos.	pos.	pos.	100
2×10^2 DNA copies/µL	pos.	pos.	pos.	pos.	pos.	100
20 DNA copies/µL	pos.	pos.	pos.	pos.	pos.	100
10 DNA copies/µL	pos.	pos.	pos.	pos.	pos.	100
5 DNA copies/µL	neg.	pos.	neg.	pos.	neg.	40

pos. – *M. dispar* DNA was detected;
neg. – *M. dispar* DNA was not detected.

Table 5
Requirements for the control results following amplification

Control	Threshold cycle (Ct) value	
	Green/FAM channel	Red/Cy5 channel
Negative PCR control	absent	absent
Negative control sample NCS	absent	≤ 35
M. dispar positive control	≤ 35	≤ 35

Analysis and interpretation of the results. Both fluorescence channels (Green/FAM and Red/Cy5) shared identical qPCR parameters: dynamic background adjustment, slope correction, 10% emission cutoff, linear scale quantification, and a detection limit of 0.05 (Fig. 2). Results are interpreted according to whether fluorescence curves intersect the threshold line, corresponding to the presence or absence of threshold cycles (Ct) in both Green/FAM and Red/Cy5 channels. These data are displayed in the corresponding amplification plots and result tables generated by the thermocycler.

The result of qPCR is considered reliable provided that the correct results are obtained for the negative PCR control,

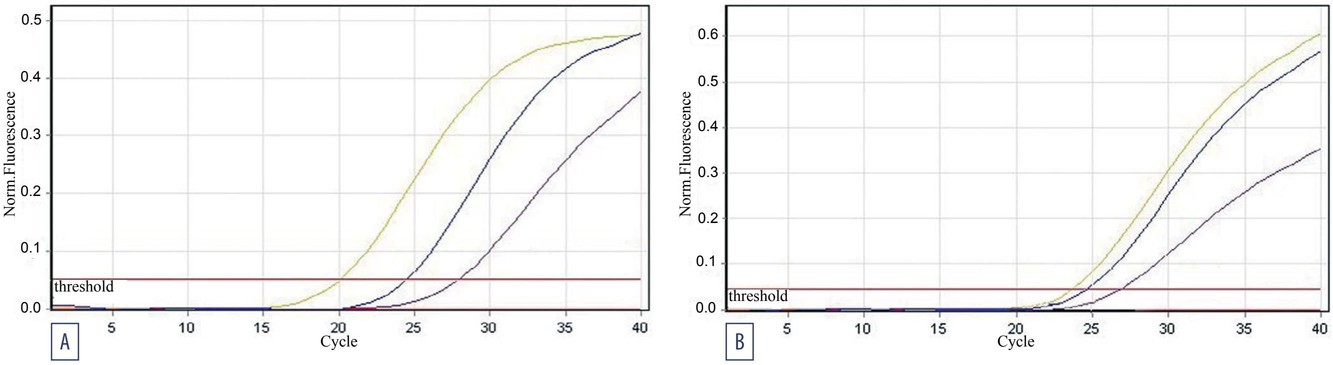

Fig. 2. Fluorescence curves: A – Green/FAM channel (M. dispar DNA); B – Red/Cy5 channel (internal control sample DNA)

the NCS (extraction control) and *M. dispar* positive control. The control requirements are described in Table 5.

The results for the tested samples should be interpreted in accordance with Table 6.

Using the developed test kit, 228 biological samples collected from cattle with respiratory clinical signs were tested, which were received by the Federal Centre for Animal Health, in 2024 from 13 regions of the Russian Federation. The results are given in Table 7.

Table 6
Interpretation of the results for the tested samples

Threshold cycle (Ct) value		Result
Green/FAM channel (*M. dispar* DNA)	Red/Cy5 channel (ICS)	
absent	≤ 35	*M. dispar* DNA not detected
≤ 35	detected or absent	*M. dispar* DNA detected
> 35	≤ 35	inconclusive[1]
absent or > 35	absent or > 35	not reliable[2]

[1] re-test relevant samples, starting from the DNA extraction stage, if the test yields the same results, it can be assumed that *M. dispar* DNA has been detected in the original sample;
[2] re-test the relevant samples, starting from the DNA extraction stage.

Table 7
Identification and detection of *M. dispar* genome in biological samples using the developed test kit in 2024

Region	Swabs (nasal, tracheal)		Pathological samples (stabilized blood, pieces of lungs, trachea and lymph nodes, pleural fluid)	
	Total number of samples	Number of positive samples	Total number of samples	Number of positive samples
Voronezh Oblast	–	–	52	0
Vologda Oblast	–	–	2	0
Vladimir Oblast	12	10	24	4
Moscow Oblast	22	8	2	2
Nizhny Novgorod Oblast	10	0	10	4
Republic of Chuvashia	10	10	–	–
Krasnodar Krai	–		2	0
Republic of Tatarstan	24	24	2	2
Ryazan Oblast	10	0	4	4
Yaroslavl Oblast	–	–	4	0
Republic of Mari El	–	–	2	2
Orenburg Oblast	6	2	8	6
Samara Oblast	16	6	6	6
Total	110	60	118	30

"–" – samples from this region were not received for testing.

It was established that *M. dispar* DNA detection rate in biological samples was 39.47%. The findings demonstrate widespread prevalence of *M. dispar* among cattle populations across Russian farms in 2024.

CONCLUSION

This study established the validation parameters and demonstrated the utility of the "MIC-DISPAR qPCR" kit for detecting *Mycoplasma dispar* DNA via real-time polymerase chain reaction. The assay exhibited high analytical specificity and sensitivity, with excellent repeatability and reproducibility across various test conditions (coefficient of variation range: 0.66–0.91%) The amplification efficiency (E) was 99.01%, and the detection limit was 100 DNA copies/reaction. These validation parameters represent essential criteria for assessing polymerase chain reaction test kit reliability.

Inclusion of an exogenous internal controls example prevents false-negative result interpretation. Using the developed test kit *M. dispar* DNA was detected in 39.47% of the tested samples received from different regions of the Russian Federation by the Federal Centre for Animal Health, in 2024, which indicates the relevance of *M. dispar* diagnostic tests. In this case, the internal controls example serves as an indicator of the extraction stage and the presence of possible inhibitors.

While the test's validation framework could be extended to include related mycoplasmas (e.g., *M. bovirhinis, M. flocculare,* and *M. ovipneumoniae*), current data lack evidence of these pathogens circulating in cattle and small ruminants within the Russian Federation. *M. ovipneumoniae* is the primary cause of mycoplasmal pneumonia in sheep and goats, and the risk of its transmission to cattle is minimal [58, 59, 60]. The demonstrated high specificity of the oligonucleotides (confirmed by BLAST analysis) supports the test kit's suitability for *M. dispar* diagnostics in cattle.

Thus, this test kit can serve as an effective tool in veterinary laboratory practice for the detection of *M. dispar* DNA in biological samples from cattle.

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