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DIFFERENTIATION OF *Mycoplasma bovis*, *Mycoplasma bovigenitalium*, *Mycoplasma californicum* AND IDENTIFICATION OF *Ureaplasma diversum* BY REAL-TIME PCR

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Abstract

Mycoplasmas and ureaplasmas are important etiological agents of mastitis, pneumonia and reproductive disorders in cattle, which cause significant economic damage to cattle farming. The most significant species are *Mycoplasma bovis*, *M. bovigenitalium*, *M. californicum* and *Ureaplasma diversum*. Commercial diagnostic PCR systems for the detection of bacteria of the genus *Mycoplasma* in different biological samples are described, but no PCR kits have been developed to address the identification of *Mycoplasma* species. In this work, real time PCR assays for differentiation of pathogenic mycoplasmas (*Mycoplasma bovis, M. bovigenitalium, M. californicum*) and detection of *Ureaplasma diversum* in biological material (semen, milk, vaginal swabs, tissues) are developed. *UvrC* gene for *M. bovis*, 16S rRNA gene for *M. bovigenitalium* and *U. diversum*, and *rpoB* gene for *M. californicum* were chosen as target genes. The PCR assays included a system of primers and probes for detection of exogenous noncompetitive internal control sample. The specificity of the developed techniques was tested on a panel of samples containing viral and bacterial pathogens causing diseases in cattle, as well as cow genomic DNA. To assess the sensitivity of each PCR assay, positive control samples were developed based on genetically engineered constructs containing the region of the corresponding specific DNA. Analytical sensitivity of the PCR assays was evaluated separately for each pathogen, for which we used 10-fold dilutions of the corresponding control samples in negative samples of biological material, i.e. semen, milk, vaginal swabs and tissues. The sensitivity (detection limit) of the assays for different types of biological species was 5×10^3 copies per ml on average. The efficiency of PCR was 99 % for *M. bovis*, 87 % for *M. bovigenitalium*, 94 % for *M. californicum*, and 98 % for *U. diversum*. A total of 410 samples of bovine semen intended for artificial insemination from local and foreign breeding centers were tested to detect *M. californicum*, *M. bovigenitalium*, *M. bovis* and *U. diversum*. DNA of *M. bovis* was found in 2.5 % of semen samples from foreign centers. In samples of Russian origin *M. bovis* DNA was not detected. DNA of *M. bovigenitalium* was identified for 60.7 % of local and 25.1 % of foreign semen samples; DNA of *M. californicum* was detected in 51.7 % and 25.1 % samples, respectively. *Ureaplasma diversum* DNA was found in 55.0 % of semen samples from Russian bulls and in 12.1 % of semen samples of foreign origin. Co-infection of *M. californicum*/*M. bovigenitalium* was detected in 97 samples (23.7 %), *M. bovigenitalium*/*U. diversum* in 86 cases (21.0 %). Simultaneous infection of *M. bovigenitalium*, *M. californicum* and *U. diversum* was observed in 52 samples (24.6 %) of semen from domestic bull sires and in 4 samples (2.0 %) from foreign breeding centers. Novel PCR assay tests can be used for monitoring of semen quality as well as control and prevention of the pathogens distribution.

Keywords: *Mycoplasma bovis*, *Mycoplasma californicum, Mycoplasma bovigenitalium*, *Ureaplasma diversum*, real-time PCR, bovine semen

Mycoplasmas and ureaplasmas are important etiological agents of mastitis, pneumonia and reproductive disorders in cattle, which cause significant economic damage to cattle farming. The most common pathogenic and clinically significant species are *Mycoplasma bovis*, *M. bovigenitalium*, *M. californicum* and

Ureaplasma diversum [1, 2].

M. bovis is one of the most dangerous pathogens, an etiological agent of upper airway diseases, pneumonia, otitis, arthritis, mastitis, endometritis, keratoconjunctivitis and other bovine conditions [3-5]. Running second after *M. bovis*, *M. californicum* inducing arthritis and pneumonia in young animals is associated with mastitis [6, 7]. *M. bovigenitalium* which also can cause bovine mastitis is detected in a reproductive tract. Additionally, it is associated with endometritis, infertility and impaired labor [8, 9]. It was demonstrated that *M. bovigenitalium* is an etiological agent of necrotic vulvovaginitis damaging cattle farms [10]. Moreover, a statistically significant correlation was found between presence of the mycoplasma in bovine semen and decreased sperm motility.

Ureaplasma diversum is another representative of *Mycoplasmataceae* that differs from *Mycoplasmae* with urea hydrolysis. At the same time, it is associated with various bovine reproductive disorders (such as granular vulvovaginitis, endometritis, salpingitis, spontaneous abortions, infertility and weak offspring) [11, 12].

Selective medium cultivation is a conventional method to detect mycoplasmas [13]. However, it has several restrictions. For example, dedicated media and microaerophilic cultivation are required for mycoplasmal growth. The testing takes 7-10 days. Along with this, another bacterial growth substantially inhibits or disables a precise identification of the etiological agent.

Nowadays, a polymerase chain reaction (PCR) with genus-specific primers is a standard method to detect mycoplasmosis. Independently from other microorganisms, it can reveal *Mycoplasmae* in various biomaterials rapidly. Nevertheless, this approach cannot differentiate a genus of the etiological agent.

Using novel PCR-based methods in the context of the paper, we detected and differentiated pathogenic *Mycoplasmataceae* in samples of cryopreserved stud bull semen used for artificial insemination in national farms. We compared mycoplasmal content in the semen products supplied by national and foreign breeding centers. Co-infection of samples with several mycoplasmal species was reported.

Our purpose was to develop methods of identification and differentiation of the most common pathogenic mycoplasmas (*Mycoplasma bovis*, *M. californicum*, *M. bovigenitalium*) and *Ureaplasma diversum* based on a real-time polymerase chain reaction.

Techniques. During our study we used vaginal swabs, milk, parenchymal organs, bovine semen and several strains such as *Mycoplasma bovis* ATCC 25523, *M. bovigenitalium* ATCC 19852, *M. arthritidis* ATCC 19611, *M. bovirhinis* PG43 ATCC 27748, *M. arginine* G230 ATCC 23838-TTR; *Histophilus somni* ATCC 700025; *Campylobacter fetus* 25936; *Brucella abortus* 82; *Yersinia enterocolitica* serotype 03; *Salmonella enterica* subsp. *enterica* Dublin 6; *Pseudomonas aeruginosa* serotype 0-17; *Staphylococcus aureus* VKPMV 6646; *Mycobacterium bovis* AN5 2/5-69-MS-07, *Mycobacterium intracellulare* 13-4; *Leptospira interrogans* Pomona VGNKI-6; *Bacillus cereus* VKPM B-8076; *Arcanobacterium pyogenes* ATCC 8164; *Neospora caninum* ATCC 50977; *Escherichia coli* 0157:H7; *Clostridium perfringens* type C; *Streptococcus pyogenes* ATCC 19615; *Candida albicans* ATCC 10231; *Aspergillus* niger ATCC 16404; *Enterococcus faecalis* ATCC 29212; Bovine Herpesvirus 1 MBA 2; Bovine Herpesvirus ATCC-VR-845; Bovine Herpesvirus 4 DN-599ATCC-VR-631; bovine diarrhea virus (DV) Oregon C24V strain; bovine DV NADL strain; bovine parainfluenza PTK 45/86 virus strain; nodular dermatitis virus DNA; bovine positive samples containing Schmallenberg disease virus RNA.

We tested 410 samples of cryopreserved bovine semen supplied by national and foreign breeding centers.

DNA was extracted with a commercial Ribo-prep kit (Amplisens, Russia). *Mycoplasmae* were detected with a Mik-Kom test system (Amplisens, Russia). A LSI VetMAX™ *Mycoplasma bovis* PCR kit (Thermo Fisher Scientific, France) was also applied.

To identify *M. bovis*, *M. bovigenitalium*, *M. californicum* and *U. diversum*, a real-time PCR was conducted with RotorGene Q (Qiagen, Germany) and CFX (Bio-Rad, USA). Amplification data were interpreted according to presence/absence of interception of a fluorescence curve and a threshold line. Amplification reaction mixture contained 10 μl of DNA matrix, 10 μl of PCR mixture 1 (6 μM specific primers, 3 μM specific probes, 3 μM primers to amplify an exogenous non-competitive inner control sample (ICS), 1.5 μM ICS probe, dNTP solution, deionized water), 0.5 μl of Taq-F polymerase, 5 μl of PCR mixture 2-FRT (Amplisens, Russia). As for *M. bovis*, *M. californicum* and *M. bovigenitalium*, we used following amplification programme: 15 min at 95 °C; 10 s at 95 °C, 20 s at 60 °C, 10 s at 72 °C (10 cycles without detection of a fluorescent signal); 10 s at 95 °C, 20 s at 55 °C, 10 s at 72 °C (35 cycles with detection of a fluorescent signal). *U. diversum* amplification program included stages as follows: 15 min at 95 °C; 10 s at 95 °C, 20 s at 55 °C, 10 s at 72 °C (10 cycles without detection of a fluorescent signal); 10 s at 95 °C, 20 s at 55 °C, 10 s at 72 °C (35 cycles with detection of a fluorescent signal).

Positive control samples (PCS) were obtained by a specific amplification product cloning in pAL2-T plasmid (Evrogen, Russia). PCR products were cloned in a pAL2-T vector by the standard manufacturer's method without pretreatment with restrictases and exonucleases. Plasmid concentration was measured with a spectrophotometer and expressed as a number of copies/ml.

Analytical method sensitivity of each pathogen was assessed separately. 10-fold dilutions of plasmids in known negative samples of semen and milk, vaginal swabs and 10% suspension of inner parenchymal organs were used as samples. Specificity was evaluated on a sample panel consisting of bovine genome DNA, as well as DNA of mycoplasmal and heterologous bacterial/viral strains inducing bovine diseases. Positive samples were verified by PCR fragment sequencing with specific primers. The sequencing was carried out with a Big Dye® Terminator v1.1 Cycle Sequencing Kit, GeneAmp PCR System 2720 amplifier (Applied Biosystem, USA) and ABI PRISM 3130 Genetic Analyzer sequencer (Applied Biosystem, USA).

Results. To detect and to differentiate mycoplasmas with molecular-genetic methods, foreign researchers use PCR schedules with various diagnostic efficiency [1, 14, 15]. To amplify *M. bovigenitalium* and *U. diversum*, most of authors apply primers selected for 16S rRNA gene and 16S-23S rRNA intergenic spacer region [16-18]. As for *M. californicum*, primers selected for *rpoB* gene are applied [1, 2]. The better part of papers is dedicated to *M. bovis* detection in various biological materials. In order to increase sensitivity and specificity, *vsp*, *fusA*, *oppD* gene primer systems are recommended [1, 14, 19]. *uvrC* gene application to *M. bovis* detection is reported [14, 15, 20]. Specific sequences selected for amplification of the genome fragment enable differentiation of *M. bovis* from *M. californicum*, *M. bovigenitalium*, *M. bovirhinis*, *M. bovoculi*, *M. dispar* and *M. agalactiae*.

Analyzing nucleotide sequences available in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) with VectorNTI Advanced 11.0 software (InforMax, Inc., USA), we proposed oligonucleotide primers and DNA probes to amplify several gene regions (i.e., *UvrC* for *M. bovis*, 16S rRNA for *M. bovigenitalium* and *U. diversum*). We identified *M. californicum* with oligonucleotide sequences reported by Boonyayatra et al. [1]. Selected primers flank

gene regions with length of 148 bp (positions nos. 697986-698133 of the GenBank CP019639.1 sequence) for *M. bovis*, 96 bp (positions nos. 504837-504932 of the GenBank CP007521.1 sequence) for *M. californicum*, 127 bp (positions nos. 131-257 of the GenBank AY974058.1 sequence) for *M. bovigenitalium*, 114 bp (positions nos. 119-232 of the GU227397.1 sequence) for *U. diversum*. We selected oligonucleotide probes to carry HEX and ROX fluorescent colorants providing simultaneous multiplex detection and differentiation of *M. californicum*/*M. bovigenitalium* and *M. bovis*/*U. diversum*. Inner control sample amplification was detected with a FAM-labeled probe in all the methods.

Oligonucleotide specificity was evaluated with Nucleotide BLAST online (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PA-GE_TYPE=BlastSearch).

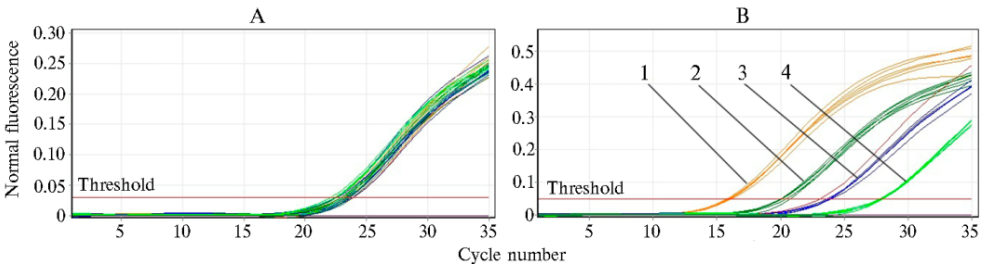
Selected nucleotides with specific targets demonstrated substantial homology and absence of significant homology with nucleotide sequences in other *Mollicutes*, viruses, bacteria or eukaryotes. Primer specificity was experimentally evidenced with a control panel including DNA of 32 strains of different microorganisms and bovine genome DNA. The panel testing showed 100% specificity.

To prevent false negative results, during DNA extraction we added an exogenous non-competitive inner control sample (ICS) amplified simultaneously with a specific target. ICS is a pAL2-T plasmid containing a synthetic DNA fragment. Added ICS enabled control of all the PCR stages for each sample.

To assess absolute primer sensitivity, we used 10-fold PCS dilutions with known concentration of a plasmid DNA containing cloned fragments of specific targets. Also, we amplified serial 10-fold PCS dilutions (5×10^5 to 5×10^2 copies/ml) in negative samples of semen, milk, bovine vaginal swabs and inner parenchymal organ suspension.

Tests were performed by different specialists using different equipment on different days. PCR efficiency was assessed automatically using RotorGene Q amplifier's software. Each sample was tested in 6 replicates. Analytical sensitivity was expressed as the lowest DNA PCS concentration providing a 6/6 positive signals in PCR.

Mean sensitivity of methods developed for different biological materials was 5×10^3 copies/ml. See milk testing data on the figure. Amplification efficiency for *M. bovis, M. bovigenitalium*, *M. californicum* and *U. diversum* was 99%, 87%, 94% and 98%, respectively.



Fluorescent signal accumulation during amplification of target DNA fragments extracted from 10-fold dilutions of positive control DNA samples in negative milk samples: A — ICS amplification (FAM fluorophore), B — *Ureaplasma diversum* amplification (HEX fluorophore); 1, 2, 3, 4 — DNA dilutions; concentration — 5×10^5, 5×10^4, 5×10^3 and 5×10^2 copies/ml, respectively.

We used developed methods to detect pathogenic mycoplasmas in samples of preserved semen collected in stud bulls. In total, 410 samples of semen supplied by national and foreign breeding centers were tested (see the Table). These samples underwent a pre-testing with a Mik-Kom test system (Amplisens, Russia) intended to detect DNA of *Mycoplasmae* in biological material [21].

***Mycoplasmataceae* detection by real-time PCR in samples of bovine semen intended for artificial insemination**

Pathogen	Breeding centers				Total (n = 410)	
	national (n = 211)		foreign (n = 199)			
	DNA detection	%	DNA detection	%	DNA detection	%
Mycoplasma spp.	182	86.3	127	63.8	309	75.4
M. bovis	0	0	5	2.5	5	1.2
M. californicum	109	51.7	44	22.1	153	37.3
M. bovigenitalium	128	60.7	50	25.1	178	43.4
Ureaplasma diversum	116	55.0	24	12.1	140	34.1

N o t e. n — number of samples..

Foreign semen demonstrated lower number of *Mycoplasmae*, in total, and pathogenic mycoplasmas, in particular, than national one. Nevertheless, *M. bovis* was detected in foreign semen only. The data were verified with a LSI VetMAX™ *Mycoplasma bovis* test system. Positive detection and differentiation of *M. bovis*, *M. bovigenitalium*, *M. californicum* and *U. diversum* were verified by sequencing.

Co-infection with several mycoplasmas was detected in 121 national (57.3%) and 31 foreign samples (15.5%). Co-infection with *M. californicum*/*M. bovigenitalium* was seen in 74 national and 24 foreign semen samples (23.7%); *M. bovigenitalium*/*U. diversum* were observed in 79 national and 7 foreign samples (21.0%). Co-infection with *M. bovigenitalium*, *M. californicum* and *U. diversum* was detected in 52 (24.6%) national and 4 (2%) foreign semen samples.

Since mycoplasmas often discharge with semen without any clinical presentations [22], pre-insemination semen test should be done to avoid infection and mastitis [23]. High incidence of bovine mycoplasmal mastitis, reproductive and respiratory disorders indicates an urgent need in verification of an etiological agent's mycoplasmal nature [1)]. According to our findings, we recommend our methods to be applied in a veterinary laboratory, to improve diagnosis and to optimize animal epidemic countermeasures, as well as to monitor quality of bovine semen intended for artificial insemination.

Thus, our methods based on a real-time polymerase chain reaction to identify and to differentiate pathogenic mycoplasmas (*Mycoplasma californicum*, *M. bovigenitalium*, *M. bovis* and *Ureaplasma diversum*), on the average, demonstrated high specificity and sensitivity (i.e., 5×10^3 of a target DNA copies/ml) in various material testings. High-degree mycoplasmal infection was detected in tested samples of frozen bovine semen intended for artificial insemination.

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