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DEVELOPMENT OF REAL-TIME PCR ASSAY FOR DETECTION OF *Anaplasma marginale*

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Abstract

Anaplasma marginale is a rickettsial pathogen responsible for bovine anaplosmosis, the acute disease in cattle herds which is associated with anemia, fever, rapid loss of milk production and weight, abortion, and, in some cases, death of the infected cattle. *Anaplasma marginale* is transmitted by ticks and biting insects. Diagnosis of bovine anaplasmosis is made by microscopic examination of blood smears stained with Giemsa stain, but this method is not useful to detect presymptomatic animals. Several serological tests have used extensively for epidemiological studies, but they do not discriminate between different *Anaplasma* species. A real-time polymerase chain reaction (PCR) combines high specificity with accurate measurement of DNA copy number and allows quantification of the targeted pathogen DNA. The goal of this study was to develop a real-time PCR assay for differential detection of *A. marginale* in the blood of cattle. The single-copy gene *msp4* was chosen as a target DNA for PCR. Msp4 is a dominant immune protein of outer membrane of all *Anaplasma* knowen to date. The primers for phylogenetic analysis in *A. marginale* based on *msp4* were reported earlier by J. de la Fuente et al. (2001), but they were not species-specific. The analysis of *msp4* gene sequence of different *A. marginale* isolates and closely related species, including *A. ovis*, revealed species-specific areas, which were used for design of primers and TaqMan probe (MSP4-F 5′-CATGAGTCACGAAGTGGCT-3′ and MSP4-R 5′-GGCACACT-CACATCAATC-3′, MSP4-probe 5′-(Cy5)-AAGGGGGAGTAATGGGAGGTAGCT-3′) for amplification and detection of 177 bp fragment of *msp4* gene by a real time PCR. In the amplified nucleotide sequences a 99 to 100 % homology to *msp4* fragments was found in different isolates of *A. marginale*. To assess analytical sensitivity of our PCR test, we used pGEM-*msp4*, a constructed recombinant plasmid with 177 bp fragment of *msp4* gene, diluted to obtain samples with 10^0-10^7 *msp4* copies. It was shown that the assay was able to detect as few as 10^2 of *A. marginale msp4* gene in the analyzed DNA sample. Analytical specificity of the developed primers and the MSP4-probe was proved in tests with DNA of sheep naturally infected by *A. ovis*, and also DNA isolated from cows with *Sanguibacter keddieii*, *Propionibacterium acnes* and *Pseudomonas aeruginosa* infection pre-detected by sequencing. In these samples no increased fluorescence characteristic of probes from animals infected by *A. marginale* was observed with no PCR products identified. Thus, the method specificity allowed to differentiate *A. marginale* and *A. ovis*. The developed method of *A. marginale* identification on the basis on amplification and detection of the *msp4* gene fragment using a real time PCR differed from known analogues with high sensitivity, rapidity and opportunity of quantitative evaluation of the bacterial load. The developed method could be used for rapid differential detection and quantification of *A. marginale* in blood samples from infected cattle for confirmation of anaplasmosis and epidemiological studies.

Keywords: *Anaplasma marginale*, *msp4* gene, cattle, diagnostics, a real-time PCR.

Bovine anaplasmosis is a vector-borne infectious disease caused by genus *Anaplasma* (order *Rickettsiales*, family *Anaplasmatacea*) rickettsia. Bovine anaplasmosis is widespread throughout the world and causes significant economic losses due to the reduced milk and meat productivity of cattle and the damages resulted from the loss of young animals and animal deaths. *Anaplasma marginale*, an obligate intracellular erythrocyte damaging parasite, is a rickettsial pathogen responsi-

ble for bovine anaplosmosis. Cattle co-infection with *A. ovis* anaplasma has also been described [1]. Infected animals are the source of anaplasmosis, and the pathogen is transmitted by about 20 species of ticks and blood-sucking insects [2, 3]. In addition, mechanical transmission of pathogen from infected animals to healthy ones is possible through contaminated zootechnical instruments.

Bovine anaplasmosis caused by *A. marginale* has been registered in many tropical and substropical countries, throughout USA and Canada, and in some European countries, mainly of the Mediterranean [4, 5]. This disease has been registered in Ukraine, Belarus, Moldova, Kazakhstan, and in the countries of Central Asia and the Caucasus. According to the Russian Federation veterinary reports on anaplasmosis, the Central, Northwestern and Volga Federal Districts are insecure [6].

Currently, microscopic and serological methods are used for the diagnosis of anaplasmosis, but their sensitivity and specificity are insufficient. The results of microscopic examination of blood smears are unreliable, especially in the early stages of infection and in cases of diseases accompanied by severe anemia [7, 8]. Serological methods based on the use of antibodies against anaplasmosis pathogen antigens are insufficiently sensitive and do not discriminate between *A. marginale* and other *Anaplasma* species [9-11]. The advantage of PCR diagnostics is its high sensitivity and specificity. It allows to detect pathogens in the earliest stages of the disease, including the latent phase, and to reliably differentiate anaplasmosis from a series of pathologies with similar clinical manifestations. According to the recommendations of the World Organisation for Animal Health (OIE) (http://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/), *A. marginale* PCR diagnostics should be performed prior to each movement of the animal to a new location, and to confirm the diagnosis. Various PCR methods to detect *A. marginale* have been described [12-22], but none of them has been fully validated.

The purpose of this study was to develop a real-time PCR assay to identify *Anaplasma marginale* in the blood of cattle.

Technique. For the selection of primers, gene sequences available in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) were used (*msp4* of various *A. marginale* isolates, as well as *A. centrale*, *A. ovis* and *A. phagocytophilum*). Conservative *msp4* sites which may serve as primers and the probe were identified using the resources of ClustalW2 server at open access (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Primer and probe species specificity was evaluated by the BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi) algorithm.

The analyzed DNA samples were isolated from whole blood of naturally infected animals (bovine cattle infected with *A. marginale*, as well as with *Sanguibacter keddieii*, *Propionibacterium acnes*, and *Pseudomonas aeruginosa*, and sheep infected with *A. ovis*). For this purpose, the Sorb-M kit (Sintol, Russia) was used according to manufacturer's recommendations.

The amplification was performed in real-time PCR in a mixture of 10 μl LightCycler® 480 Probes Master PCR reagent (Roche, Switzerland); MSP4-F 5'- CATGAGTCACGAAGTGGCT-3' primer (0.5 μM), MSP4-R 5'-GGCACACTCACATCAATC-3' primer (0.5 μM); MSP4-probe 5'-(Cy5)-AAGGGGGAGTAATGGGAGGTAGCT-3' fluorescently labeled probe (0.1 μM); 3 μl of DNA; total reaction volume was 20 μl. PCR was performed using a LightCycler 96 instrument (Roche, Switzerland) under the following conditions: initial denaturation for 2 min at 95 °C; 45 cycles of 15 sec at 95 °C, 15 sec at 58 °C, 15 sec at 72 °C. The fluorescence signal was recorded for the Cy5

channel. PCR results were also assessed by electrophoresis in 2 % agarose gel.

The *msp4* gene fragment of 177 bp produced in PCR was purified using the GeneJET PCR Purification Kit (Life Technologies, USA), ligated into pGEM-T vector (Promega, USA) and cloned in *Escherichia coli* DH5 cells. To identify the colonies of transformants containing the pGEM-*msp4* plasmid, PCR was performed using a pair of standard primers for M13 sequencing (SibEnzyme, Russia) with subsequent analysis of the amplification products by electrophoresis in 2 % agarose gel.

Target colonies were incubated overnight at 37 °C in 2 ml of liquid LB medium, containing ampicillin at 100 μg/ml concentration. To purify plasmid DNA, GeneJET Miniprep Kit (Thermo Fisher Scientific, США) was used. Plasmid DNA concentration was estimated using PicoGreen® dsDNA Assay Kit (Thermo Fisher Scientific, USA) with a QuantiFluor-ST fluorimeter (Promega, USA). Sequencing of the resulting pGEM-*msp4* plasmids was performed according to the Sanger method with ABI Prism Big Dye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and an Applied Biosystems 3130 genetic analyzer (Life Technologies, USA). To determine PCR analytical sensitivity, a series of 10-fold dilutions of the pGEM-*msp4* plasmid was made and the samples containing 10^0-10^7 *msp4* gene copies were obtained. Estimation of PCR analytical sensitivity was performed in triplicate.

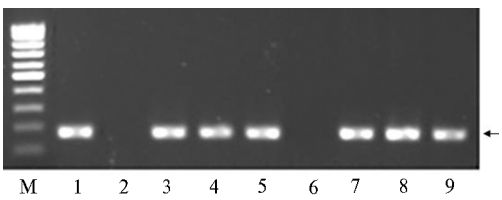

Fig. 1. Electrophoretic separation of DNA produced in PCR using the original primers (MSP4-F and MSP4-R) and DNA extracted from the blood of infected (1, 3-5, 7-9) **and uninfected with *Anaplasma marginale*** (2, 6) **cows:** M — DNA size marker (100-1000 bp, Dialat Ltd., Russia). An arrow marks the fragment of 177 bp.

Results. In this study, the primers and probe for the amplification and detection of *A. marginale msp4* (major surface protein 4) gene fragment for real-time PCR were designed. Msp4 is an immunodominant outer membrane protein found in all currently known genus *Anaplasma* rickettsia [5, 23]. According to whole-genome sequencing, *msp4* is presented by one copy only in *A. marginale* [24]. The *msp4*-based primers for phylogenetic analysis of *A. marginale* have been proposed earlier by J. de la Fuente et al. [13], but they are not species specific for this anaplasma since they are 100 % identical to the portions of *A. ovis msp4* gene, whereby they can not be used to differentiate these two pathogens. The *msp4*-based primers have also been proposed for the differential detection of *A. marginale* and *A. ovis* by classic PCR with the identification of amplification products by electrophoresis in agarose gel [17]. The real-time PCR advantage compared to classical PCR is the registration of DNA accumulation directly during PCR, i.e. the PCR result detection as an additional analysis phase is absent. This reduces the time of study considerably, while the combination of primers and species specific fluorescently labeled probe improves the reliability of correct pathogen identification.

The selected MSP4-F and MSP4-R primers were used for PCR in which bovine DNA from *A. marginale* infected animals served as a template, that was confirmed by the results of earlier genome sequencing of bovine genome fragments [25], and PCR with AmargMSP4Fw 5'-CTGAAGGGGGAGTAATGGG-3' and AmargMSP4Rev 5'-GGTAATAGCTGCCAGAGATTCC-3' primers for *msp4* in *A. marginale*, according to A. Torina et al. [17] was conducted. As a result of PCR with the MSP4-F and MSP4-R primers designed by

us (Fig. 1), DNA fragments of 177 bp were produced and sequenced.

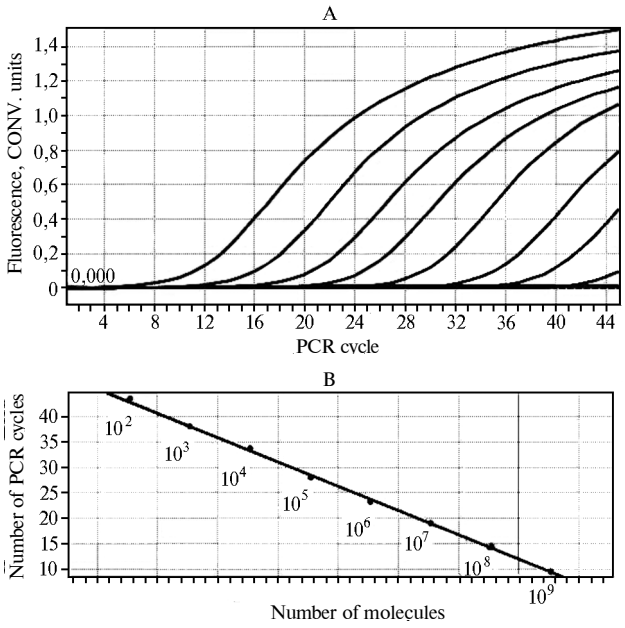

Fig. 2. Curves of fluorescence changes in real-time PCR for 10-fold serial pGEM-*msp4* plasmid delutions (A) **and estimation of sensitivity threshold (dependence of the required number of PCR cycles on the initial number of *msp4* copies in the reaction mixture)** (B). Light-Cycler 96 (Roche, Switzerland; the fluorescence signaal was recorded for the Cy5 channel).

In the amplified nucleotide sequences a 99 to 100 % homology to *msp4* fragments was found in different isolates of *A. marginale*. The same MSP4-F and MSP4-R primers and the MSP4-probe were used in real-time PCR. In all DNA samples from infected cows, an exponential increase in fluorescence was observed.

To test the specificity of MSP4-F and MSP4-R primers and MSP4 probe, DNA samples from sheep infected with *A. ovis* rickettsia were used, which was found as a result of PCR with AovisMSP4Fw 5′-TGAAGGGAG-CGGGGTCATGGG-3′ and AovisMSP4Rev 5′-GAGTAATTGCAGCCAGG-GACTCT-3′ primers for A. *ovis msp4* [17], as well as bovine DNA samples which according to sequencing [25] contained *Sanguibacter keddieii*, *Propionibacterium acnes* and *Pseudomonas aeruginosa* bacterial DNA. Unlike the samples from *A. marginale* infected animals, no increases in fluorescence were observed in these DNA samples in real-time PCR and no PCR products were identified under electrophoretic separation indicating the species specificity of primers and probe.

The pGEM-*msp4* plasmid with the *msp4* gene fragment of 177 bp (standard) was constructed in estimating PCR analytical sensitivity, and the samples containing 10^0-10^7 copies of msp4 gene fragment were obtained by 10-fold serial dilutions of the standard. The results of PCR with these samples as template and combination of the primers and the probe developed by us demonstrated that the method sensitivity makes it possible to detect 10^2 and more *msp4* copies in the test sample volume (Fig. 2), that is, given the single copy of this gene [24], from 100 molecules of *A. marginale* DNA.

It should be noted that our method of *A. marginale* detection differs from the existing analogues [13, 17] due to high specificity, rapidity and the possibility of quantifying bacterial load.

Thus, we have developed a pair of primers and a probe for the *msp4* gene for differential detection of *Anaplasma marginale* rickettsia in bovine blood by real-time PCR. This method sensitivity makes it possible to detect from 10^2 *msp4* copies in the *A. marginale* DNA sample volume, and its specificity is sufficient to reliably differentiate *A. marginale* and *A. ovis*. So the suggested method is highly specific unlike those described in publications. Moreover, it makes it possible to quantify the bacterial load, while the procedure requires less time. Therefore, it can be used, if necessary, for the

prompt differential detection and quantification of *A. marginale* in the blood samples of infected cattle to confirm the diagnosis and perform epidemiological monitoring of anaplasmosis.

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