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# Development of a real-time PCR for the identification of *Anaplasma marginale* in cattle

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**Abstract**

*Anaplasma marginale* is a gram-negative, obligate intracellular bacterium that infects cattle, buffalo, deer, and antelope, causing significant economic losses worldwide. In recent years, due to an increase in the area of uncultivated land and uncontrolled use of pastures, there has been a tendency for a sharp increase in the population of blood-sucking ticks - carriers of blood-parasitic animal diseases. In this regard, the development of molecular genetics methods for the diagnosis of blood parasitic diseases is relevant for taking effective measures to prevent the spread of tick-borne infections. The lack of sensitivity and specificity of the microscopic and immunological diagnostic methods used, as well as the tendency for a sharp increase in the population of blood-sucking ticks, aggravates the epizootological situation. As part of this research, a real-time PCR test system for the identification of *Anaplasma marginale* was developed. Species-specific primers and a fluorescence-labelled TaqMan probe were developed for the highly conserved gene of the heat shock family protein *groEL.* The developed real-time PCR test system showed high specificity and sensitivity, allowing the detection of the *groEL* gene of *A. marginale* at the level of 8 copies in the reaction. The developed real-time PCR test system can be used for early diagnosis of anaplasmosis in cattle and conducting monitoring studies.

**Keywords:** *Anaplasma marginale*; cattle; real time PCR; qPCR; *groEl.*

## Introduction

Anaplasmosis an infectious disease of animals caused by rickettsias of the genus *Anaplasma.* The disease is caused by gram-negative obligate intraerythrocytic parasites of the family *Anaplasmataceae* of the genus *Anaplasma* of the following species: *A. ovis, A. bovis, A. capra, A. marginale, A. centrale, A. phagocytophilum, A. platys*. Various members of the *Anaplasma spp.* can infect large and small livestock, horses, deer, antelope, moose, dogs, cats, as well as humans [1, 2].

Anaplasmosis is one of the tick-borne infections of economic importance to agriculture, leading to loss of meat and dairy productivity, and in severe cases lead to death [3]. In addition, weakened animals are more susceptible to other infectious or parasitic diseases, which increases the mortality of livestock. The most acute pathological process is characteristic of the species A. marginale, during which about

70% or more of red blood cells are affected, which can lead to the death of 30-50% of cattle [4, 5].

*A. marginale* is widespread all over the world, especially in tropical and subtropical regions. The disease is accompanied by hemolytic anemia, fever, disorders of the gastrointestinal tract and respiratory organs, and weight loss [6]. The main vectors of anaplasmosis are ticks of the species *Ixodes, Rhipicephalus, Haemaphysalis, Dermacentor,* etc., as well as mosquitoes, horseflies and other blood-sucking insects, which means that the disease is naturally localized and can quickly spread to neighboring farms [7, 8].

In the southern regions of Kazakhstan, *Anaplasma marginale* predominates among tick-borne infections of cattle, accounting for 48.9% among tested animals [9]. At the same time, effective measures to control the spread of infection are not taken, mainly due to the complexity of differential diagnosis and the lack of highly sensitive and specific test systems, which contribute to the spread and chronic course of the disease with periodic relapses.

Currently, the most commonly used methods for diagnosing anaplasmosis are Giemsa staining of smears and serological methods. Accuracy of diagnosis is often hampered by insufficient numbers of circulating infected cells, especially during the prodromal period or in cases of latent animal carriers. False-positive results are also possible with Giemsa staining due to staining artifacts and Heinz and Howell-Jolly bodies similar to anaplasma-like structures [10].

In the early acute phase of infection, serological tests are of limited value due to the lack of detectable antibodies. In addition, the difficulty of differential diagnosis in serological studies is determined by similar symptoms and possible cross-reactions not only between similar types of anaplasmas, but also with other tick-borne infections: babesiosis, borreliosis, ehrlichiosis, Lyme disease, rickettsiosis [11]. A seronegative course of anaplasmosis is possible, which prevents timely detection and contributes to the further spread of infection through common pastures and grazing areas.

Despite the fact that a number of different diagnostic methods have been developed for intraspecific identification of anaplasmosis (complement-enzyme linked immunosorbent assay, card agglutination test, complement fixation test, loop-mediated isothermal amplification, reverse line blot, PCR, nested PCR, quantitative PCR), most of them have insufficient sensitivity and specificity [12]. The best results today are shown by PCR; however, universal and generally accepted methods are not suitable for intraspecific diagnosis of anaplasmosis. PCR methods based on amplification of the 16S rRNA gene have already been used in the analysis of the *Anaplasma* genus. Due to the high degree of similarity between the species *A. marginale, A. centrale* and *A. ovis*, this method does not allow them to be differentiated, which is due to the low rate of evolutionary changes in the 16S rRNA gene [13].

As part of these studies, the groEL gene was selected as the most suitable for single locus genotyping. It is highly conservative, but contains variable regions that allow it to be used to differentiate closely related species [14].

The purpose of this work was to develop a PCR protocol for the detection and species identification of *Anaplasma marginale*.

## Materials and methods

### Ethical approval

This study was approved by the local ethics committee in the National Center for Biotechnology (Protocol № 2 dated 04 April, 2022). The respective cattle owners gave their approval for sampling. No animal was harmed during the sampling.

Sampling and sample preparation

To screen for anaplasmosis among cattle, 962 whole blood samples were collected from the Turkestan region. Blood was collected in EDTA vacutainers and transported to the laboratory at 4 °C for 48 hours. For lysis of erythrocytes, a lysing buffer (1.5 M $NH_4Cl$ (PanReac AppliChem, Darmstadt, Germany), 100 mM $NaHCO_3$ (Thermo Fisher Scientific, Fair Lawn, USA), 10 mM EDTA (BioRad, Richmond, USA), $H_2O$) was used in a ratio of 1:3, and after stirring and 5 minutes' incubation at room temperature, centrifugation was carried out for 5 minutes at 13,000 rpm, followed by removal of the supernatant. The pellet was stored at minus 80 °C.

*DNA isolation*

DNA isolation was carried out on the basis of sorbent DNA binding. The precipitate was dissolved in 150 µl of lysing solution 1: 0.4 M NaCl (Titan Biotech Ltd., Rajasthan, India), 10 mM Tris-HCl pH 8.0 (BioRad, Richmond, USA), 2 mM EDTA, 5% SDS (Sigma-Aldrich, Darmstadt, Germany), 1 mg/ml of proteinase K (Magen, Guangzhou, China). Samples were incubated at 37 °C for 4 hours and at 50 °C for 50 minutes. 500 µl of lysis buffer 2 (3.2 mM GuaSCN (PanReac AppliChem, Darmstadt, Germany), 20 mM Tris-HCl (pH 7.4), 50 mM EDTA, 4% Triton X-100 (Amresco, Solon, USA) and 30% isopropanol (Sigma-Aldrich, St. Louis, USA) was added and incubated at 60 °C for 10 minutes. At the next stage, a sorbent (3:7 $SiO_2$ (Alfa Aesar, Kandel, Germany) and Celitre 545 AW (Sigma-Aldrich, St. Louis, USA) respectively) in an amount of 70 µl was added to the test tube, the samples were incubated for 5 minutes at 60 °C and centrifuged for 1 minute at 2 000 rpm. Purification of sorbent-bound DNA was carried out at 5 000 rpm for 1 minute, first in 300 µl of wash buffer 1 (3.2 M GuaSCN, 0.1 M Tris-HCl), then twice in 500 µl of wash buffer 2 (75% $C_2H_6O$ (DOSFARM, Almaty, Kazakhstan), 10 mM Tris-HCl). The sorbent was dried for 10 minutes at 60 °C, and DNA was eluted in 200 µl of 0.1xTE buffer (PanReac AppliChem, Darmstadt, Germany) for 10 minutes at 60 °C. Concentration measurements were carried out spectrophotometrically using Nanodrop-1000 (Thermo Fisher Scientific, Wilmington, USA).

*Identification and species identification of Anaplasma spp.*

The identification and species identification of *Anaplasma spp.* was carried out by amplification and sequencing of the *groEL* fragment with the following primers: anapl_F-1393 5'-aaggatggatayaaggtmatgaa-3' and anapl_R1852 5'-cgcggwcaaactgcatac-3'. The reaction mixture was prepared in 30 µl and contained 10 mM Tris-HCl (pH 8.8 at 25 °C), 50 mM KCl, 0.08% (v/v) Nonidet P40, 300 nM of each primer, 2.5 mM $MgCl_2$, 200 nM of each dNTP, 2 units of Taq DNA Polymerase (Biolabmix, Novosibirsk, Russia) and 5 µl of DNA. The PCR cycling program was carried out on the MasterCycler ProS (Eppendorf, Hamburg, Germany): 1 denaturation cycle for 5 minutes at 95 °C, 35 amplification cycles (95 °C - 30 s, 60 °C - 40 s, 72 °C - 50 s), 1 final extension cycle for 5 minutes at 72 °C. The products were detected on a 1,5% agarose gel with ethidium bromide as intercalating agent. Visualization was performed on the GelDoc system (BioRad, Hercules, USA) using Image Lab Software (BioRad).

Species identity was determined by Sanger sequencing. Magnetic particles were used as previously described [15] for purification of PCR products. The BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Vilnius, Lithuania) was used for sequencing according to the manufacturer's instructions. Fragments were separated using a capillary genetic analyzer 3730xl (Applied Biosystems, Carlsbad, USA). The resulting contigs of the forward and reverse primers were analyzed in the SeqMan program (Lasergene, DNASTAR) and identified using the Nucleotide BLAST tool in the GenBank NCBI database.

*Preparation of plasmids*

Plasmid DNA was used to determine PCR sensitivity. A 445 bp fragment of the *groEL* gene was cloned into the pGEM-T plasmid using the pGEM-T Easy Vector Systems I kit (Promega, Madison, USA) according to the manufacturer's instructions and transformed into chemocompetent E. coli DH5α cells. Plasmid DNA was isolated using the Wizard SV 96 Plasmid DNA Purification System Kit (Promega, Madison, USA) according to the manufacturer's instructions. DNA concentration was measured on a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, USA) using Qubit dsDNA HS Assay Kits (Invitrogen, Eugene, USA).

*Selection of qPCR primers*

The selection of primers and fluorescent probe for A. marginale was based on the alignment of the nucleotide sequence of the groEL gene in BioEdit software (Hall T. A). The groEL gene sequences for alignment were obtained from the NCBI GenBank database and included members of the following species: *Anaplasma marginale, Anaplasma centrale, Anaplasma phagocytophilum, Anaplasma ovis, Anaplasma bovis, Anaplasma platys, Anaplasma capra.*

*qPCR temperature optimization*

qPCR was carried out using a temperature gradient from 58 to 64 °C to optimize amplification efficiency. The reaction was performed in 3 samples of each species: *A. marginale, A. centrale, A. ovis,* and an *E. coli* sample as a negative control. Amplification was carried out in a CFX96 instrument (BioRad,

Singapore) using a BioMaster UDG HS-qPCR kit (Biolabmix, Novosibirsk, Russia) according to the manufacturer's instructions. The PCR mix in a volume of 25 µl is included: 1x ready-to-use BioMaster UDG HS-qPCR (Biolabmix, Novosibirsk, Russia), 300 nM of each primer, 300 nM fluorescent probe and 5 µl of DNA. qPCR protocol program: 1 cycle of anti-contamination treatment for 2 minutes at 50 °C, 1 cycle of pre-denaturation for 5 minutes at 95 °C, 45 cycles of amplification at 95 °C - 15 s and 58-64 °C - 60 s. Fluorescence results were considered at the annealing/elongation stage, excluding the first 10 amplification cycles. Analysis was performed using BioRad CFX Manager software.

*Determination of qPCR sensitivity and specificity*

Intraspecific specificity was assessed on 32 samples of *A. marginale*, 24 samples of *A. centrale* and 24 samples of *A. ovis* obtained by screening PCR in cattle and identified by Sanger sequencing. In order to determine the interspecies specificity, 3 samples of *A. marginale* were used as positive controls, 3 samples were used as negative controls and 90 species of bacteria were selected from the internal collection, including the following species: *Acinetobacter junii, Aerococcus urinaeequi, Alcaligenes (A. aqualitis, A. faecalis), Arthrobacter polychromogenes, Atopobium vaginae, Bacillus (B. aerius, B. amyloliquefaciens, B. cereus, B. licheniformis, B. pumilus, B. sonorensis, B. velezensis, B. wiedmannii), Bacterium spp., Bordetella bronchiseptica, Brevibacillus borstelensis, Brucella (Br. abortus, Br. Melitensis), Campylobacter ( C. coli, C. jejuni, C. rectus, C. showae), Clostridium (Cl. chauvoei, Cl. Haemolyticum), Delfitia tsuruhatensis, Enterobacter cloacae, Enterococcus (E. durans, E. faecalis), Erwinia (Er. endophytica, Er. Tasmaniensis), Exiguobacterium (Ex. alkaliphilum, Ex. aurantiacum, Ex. profundum, Flavobacterium sp, Halomonas nitritophilus, Klebsiella (Kl. michiganensis, Kl. oxytoca, Kl. Pneumonia), Lactobacillus (L. crispatus, L. gasseri, L. iners, L. jensenii, L. paracasei, L. paraplantarum, L. pontis), lactococcus garvieae, Lysinibacillus (L. alkalisoli, L. xylanilyticus), Macrococcus (M. canis, M. caseolyticus, M. equipercicus), Mannheimia (M. granulomatis, M. varigena), Massilia putida, Moraxella bovoculi, Ochrobactrum (O. anthropi, O. thiophenivorans), Paenibacillus (P. mucilaginosus, P. sordellii), Pantoea agglomerans, Pasteurella multocida, Pediococcus acidilactici, Propionivibrio limicola, Pseudomonas (Ps. mandelii, Ps. mucidolens, Ps. peli, Ps. putida, Ps.silesiensis, Ps. syringae), Rhizobium (R. nepotum, R. pusense), Rhodococcus (Rh. corynebacterioides, Rh. kroppensteti, Rh. opacus), Salmonela enteretidis, Serratia (S. liquefaciens, S. marcescens, S. proteamaculans), Shigella (Sh. flexneri, Sh. sonnei), Solibacillus isronensis, Staphylococcus (S. chromogenes, S. epidermidis, S. haemolyticus, S. intermedius), Streptococcus Str. criceti, Str. pluranimalium, Str. salivarius, Xenophilus arseniciresistens.*

The sensitivity of the reaction was determined by diluting plasmid DNA with an initial concentration of 1.845 ng/µl containing $4.96\times10^8$ copies of the target gene. The copy number was calculated using the online calculator «https://www.technologynetworks.com/tn/tools/copynumbercalculator». A qPCR reaction was set up using 5 µl of DNA, first diluted to $4.19\times10^6$ copies for the first row of wells in 3 replicates, followed by 4-fold dilutions to 16 copies and then 2-fold dilutions to 1 copy.

**Results**

*Primer selection*

The results of the *groEL* sequence alignment revealed a highly conserved region for *A. marginale.* The following primers and probe were selected A. marg-U 5'-gatgagattgcacaggttgct-3', A. marg-R 5'-tcctcaaccgttattaccccg-3', An. marg-PF FAM-tgccaacttcccttacgcactgtgc-BHQ1.

As shown in Figure 1, the selected forward and reverse primers have variable nucleotides on the 5' sequences to other *Anaplasma* species, which will not allow them to anneal.

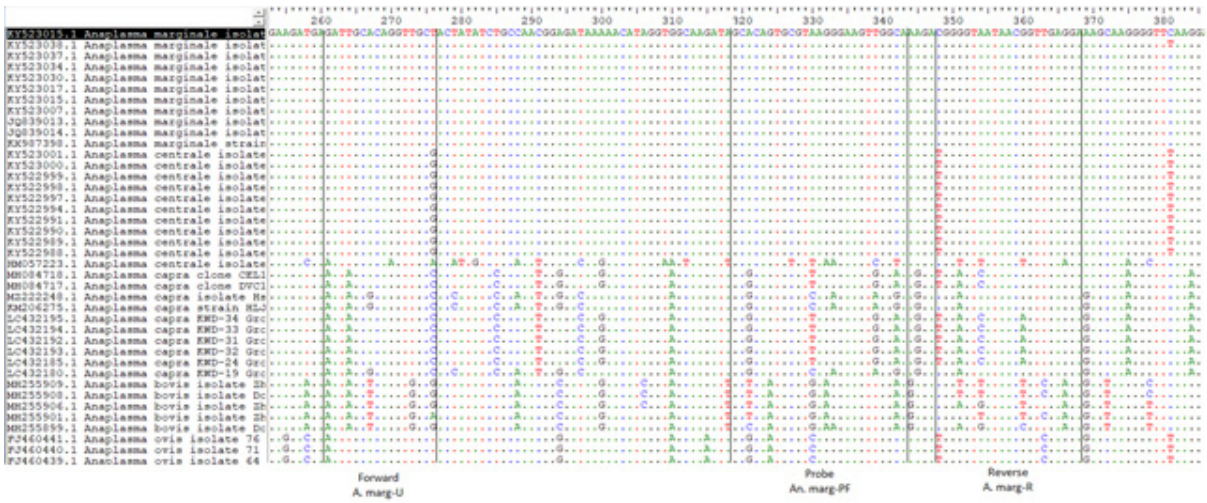


Figure 1 – Selection of primers and fluorescent probe

Testing with PrimerSelect (Lasergene, DNASTAR) did not reveal the presence of refractory dimers at the 3' ends. The annealing temperature in NCBI Primerblast was 58.91 °C and 59.52 °C for the forward and reverse primers, respectively.

*qPCR optimization*

According to the qPCR results shown in Figure 2, there was no non-specific annealing of primers for other *Anaplasma* species and E. coli, and the optimal temperature was 60 °C. This temperature showed the maximum level of fluorescence when reaching a plateau and a comparable value of the threshold cycle (Ct) with the results at temperatures of 61-64 °C for all three *A. marginale* samples. Lowering the temperature below 60 °C increased Ct by 1 value and decreased the fluorescence intensity. At higher temperatures, no significant differences in Ct were detected, but a decrease in fluorescence level was recorded when the curve reached a plateau.

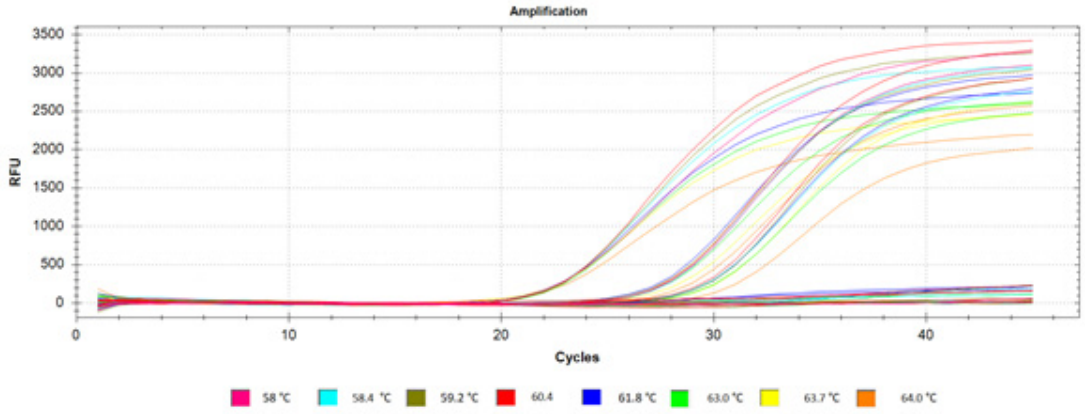


Figure 2 – Graphs of amplification curves in a temperature gradient of 58-64 °C

*Testing qPCR specificity and sensitivity*

Specificity and sensitivity were assessed at the optimal selected temperature of 60 °C. As a result of qPCR, specific annealing and amplification were only detected in samples containing *A. marginale*, as shown in Figure 3. The negative control in the form of *E. coli, A. ovis* and *A. centrale* species did not show any increase in the amplification curves.

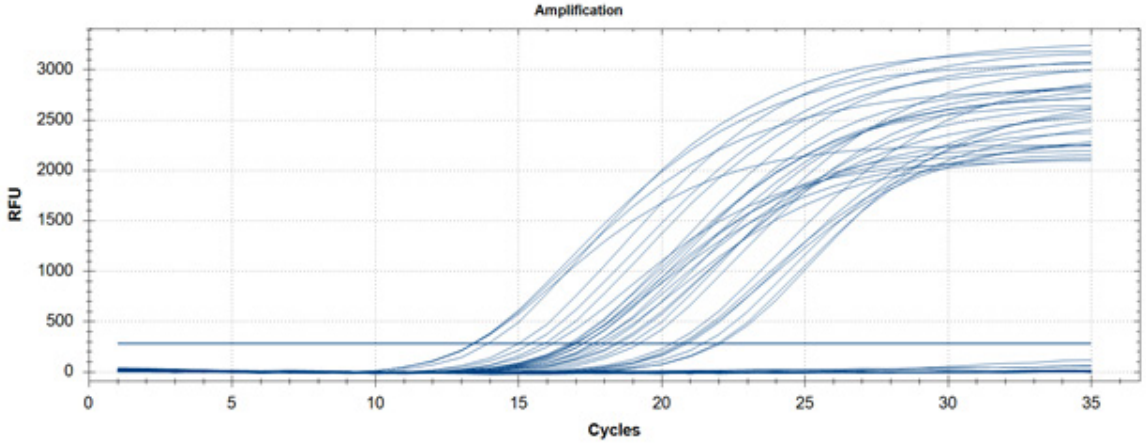


Figure 3 – Assessment of primer species specificity

The qPCR results shown in Figure 4 confirmed the absence of specific annealing to 90 other bacterial species and the presence of fluorescence curves only on the target organism.

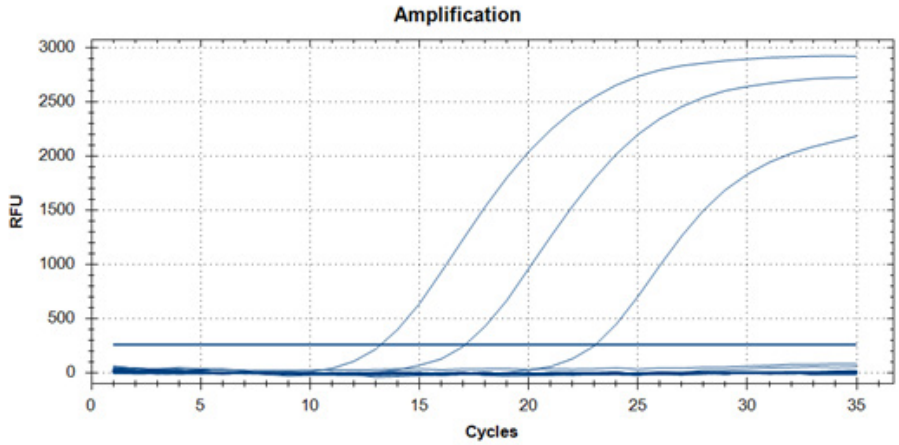


Figure 4 – Testing non-specific annealing of primers for other types of bacteria

Sensitivity testing was carried out on a plasmid containing the *A. marginale groEL* gene. The minimum sensitivity threshold for qPCR was found to be 8 copies per reaction; there was no increase in the fluorescence curves at lower copy numbers. The results are shown in Figure 5.

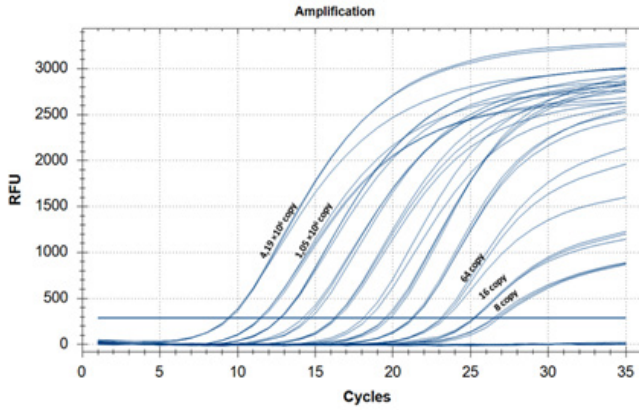


Figure 5 – Amplification curves for sensitivity assessment

**Discussion**

As a result of this study, a real-time PCR (qPCR) test system was developed for the identification of *A. marginale* with high specificity, allowing differentiation from other species of *Anaplasma spp.* The sensitivity of the method was 8 copies per reaction.

In molecular diagnostics, *Anaplasma spp.* are targeted for species identification. are genes for heat shock protein (*groEL*), disulfide oxidoreductase (*dsd*), citrate synthase (*gltA*) and basic surface proteins (*msp1, msp1a, msp2, msp4, msp5*) [16].

Previously, qPCR was used for the identification of *A. marginale* in the work of G. Carelli et al. where a comparison was made with reverse line blot (RLB) and nested PCR methods. The two-step nPCR method and the faster qPCR method for the multilocus *msp1β* gene showed the same sensitivity of 10 DNA copies and allowed the identification of positive samples not detected by the RLB method [17]. M. Chaisi's results confirmed that the sensitivity of qPCR was superior to RLB, 25 DNA copies versus 2500 copies. They also found that there was variability in the target region of the *msp1β* gene, leading to false negatives when using nPCR [18]. R. Giglioti compared the loop-mediated isothermal amplification (LAMP) they developed with qPCR on a region of the *msp1β* gene. The sensitivity of qPCR was 21 copies per 1 μL, which is 10 times higher than the LAMP [19].

A. Arkhipova et al. used the msp1α gene as a target gene for the development of qPCR; the sensitivity of the method they developed was one copy of the gene, and the specificity was sufficient for differentiation from other *Anaplasma species* [20]. Surface proteins such as *msp1β* and *msp1α* are often used to create PCR test systems, but the disadvantage of these tests is the rapid variability of surface proteins, which requires careful selection of primers for a specific geographic region [21].

In the studies by S. Kovalchuk et al, a qPCR based on the single locus *msp4* gene was developed for the identification of A. marginale, which allows differentiation of *A. marginale* from *A. ovis* with a sensitivity of $10^2$ DNA copies [22]. Although the difference in identity of the msp4 gene is sufficient to differentiate A. marginale from A. centrale at 83%, the lack of data on the testing of this assay for *A. centrale* may lead to false positive results [23].

In studies by G. Picoloto et al. qPCR and standard PCR targeting the *msp5* gene showed earlier detection of *A. marginale* in calves, while smear analysis confirmed the presence of infection only 5 and 26 days after tick exposure. This study also showed better sensitivity of qPCR, identifying 7 positive reactions from 43 deer samples versus 1 positive reaction by standard PCR [24]. Similar results were obtained by G. Bacanelli, who detected 83.3% of infected animals on day 7 using qPCR for the msp5 gene and 16.7% using standard PCR [25]. The sensitivity of qPCR was also shown to be better than the indirect enzyme immunoassay indirect ELISA in a comparative analysis by A. Ali Turi et al. where qPCR detected 34.8% of infected animals versus 28.7% detected by iELISA [26].

Real-time PCR for the identification of *Anaplasma spp.* has not been used in Kazakhstan before, and its advantage is the speed of analysis, the elimination of additional detection steps, and the ability to determine the carrier status of an infected animal. qPCR requires less labour and greatly simplifies the process compared to traditional methods for identifying *Anaplasma marginale*, and simultaneous detection during thermal cycling significantly speeds up the process compared to standard PCR. The qPCR test system we developed, based on the *groEL* gene, showed high sensitivity and specificity, not inferior to similar developments, and may in the future replace the immunological analysis methods used today.

**Conclusion**

A highly sensitive and specific real-time PCR test system for species identification of *A. marginale* has been developed. Highly specific primers and a probe for the *groEL* gene were developed to differentiate *A. marginale* from the closely related species *A. centrale* and *A. ovis*. The PCR protocol was optimized and the optimal primer annealing temperature was established. The sensitivity of the test system was 8 copies per reaction. This PCR test system can be used for effective monitoring in epizootically disadvantaged regions of Kazakhstan; identification of carriers will ensure control of the spread of *A. marginale* and timely implementation of preventive measures.

**Authors' Contributions**

Conceptualization, K.M. and A.Sh.; methodology K.M. and A.Sh.; validation, A.O., A.K. and A.D.; formal analysis, K.M., A.O. and N.T.; investigation, K.M.; resources, K.M.; data curation, A.Sh. and K.M.; writing–original draft preparation, A.O., M.K., N.T., A.D., A.K., A.Sh., M.F., E.Sh. and M.K.; writing–review and editing, K.M and A.Sh.; visualization, A.O. and M.K.; project administration, K.M.; funding acquisition, K.M. All authors have read and agreed to the published version of the manuscript.

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