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OPTIMIZATION OF PCR PROTOCOL TO IDENTIFY *THEILERIA ANNULATA*

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ABSTRACT

Theileriosis is a blood-parasitic disease of domestic and wild animals caused by *Theileria annulata*. Although the disease course varies in severity, it is most often fatal. Theileriosis is endemic to the southern regions of Kazakhstan, and the main means of combatting the disease are accurate diagnosis and appropriate treatment. In this regard, the timely detection of infection is a critical step in initiating preventive measures. Polymerase chain reaction (PCR) allows for the detection of latent carriage of the parasite with higher sensitivity than classical microscopic detection. The aim of this work was to determine the optimal parameters of PCR for detecting *T. annulata* DNA using the enolase (ENO) gene as a diagnostic target. Selected primers at the estimated rates of the PCR revealed the presence of *T. annulata* in 4 of 18 DNA samples isolated from ticks. Subsequent optimization of the conditions increased the efficiency of PCR. The specificity of the developed protocol was confirmed by direct sequencing of known positive samples. This optimized method is expected to improve diagnosis at an early stage of infection to allow for timely treatment and a better outcome.

Key words: theileriosis, ixodid ticks, PCR, sequencing, phylogenetic analysis

INTRODUCTION

Theileriosis - an animal disease caused by the hemoparasites of the genus *Theileria* [1]. *Theileria* is a genus of parasitic protozoa of the spore family of *Theileriidae*. They parasitize in the cells of the reticuloendothelial system and in animal red blood cells. A number of *Theileria* species that cause theileriosis disease are: in cattle - *Theileria annulata, Theileria parva, Theileria mutans, Theileria dispar, Theileria sergenti*; in sheep and goats - *Theileria ovis, Theileria hirci, Theileria recondita*; in reindeers - *Theileria tarandi-rangiferis*; in deers - *Theileria cervi.* Blood-sucking ixodid ticks are carriers of theileria pathogenes [2]. These pathogens have several characteristics that make them unique among the most known *Apicomplexa*. *Theileria* genus is characterized by infection of leukocytes with sporozoites, maturation of schizonts in merozoites, and subsequent infection of red blood cells with the formation of pyroplasmas [3]. Unlike many apicomplexas, these pathogens are located in the host cytosol, and not inside the parasitophorous vacuole. *Theileria* are two-host parasites: the first definitive host is invertebrates: blood-sucking ixodid ticks, in them pathogens breed by schizogony and sexually. The second intermediate host is vertebrates: cattle, buffaloes, zebu, yaks; in them *Theileria* pathogens reproduce asexually: by multiple division (schizogony) and simple division (monotomy) [4, 5, 6].

The occurrence of the disease is limited by the geographical distribution of the respective tick species. In some endemic areas, livestock has a fairly high degree of innate resistance. Mortality in these areas is relatively low, but imported cattle are particularly vulnerable [7]. In Kazakhstan, theileriosis is recorded in the southern regions: South Kazakhstan, Zhambyl, Kyzylorda regions,

which are permanently disadvantaged localities [8, 9, 10, 11].

As a rule, the diagnosis of hemoparasites is based on an analysis of a blood smear. [12]. However, this method is non-specific, accompanied by some technical problems and, in some cases, is not possible due to the low level of parasitemia [13]. Molecular methods such as polymerase chain reaction (PCR) are much more sensitive and help to differentiate the infection at the level of species and subspecies [14], to detect theileriosis in the clinical stage (pyroplasma in red blood cells) and the carrier stage (schizont, myrogonium, myrozoyte) in lymphocytes [15].

The endemicity of theileriosis in the southern regions of Kazakhstan against the background of free movement of animals with low control of blood-parasitic diseases creates the risk of the formation of new pesthole and aggravation of the situation in endemic regions. Diagnosis is an essential element in epizootological monitoring and disease control. In this regard, the goal of this work was to develop a PCR protocol to detect *Theileria annulata.*

Materials and methods

18 samples of ixodid ticks collected as the studied material in May 2019 from cows contained in private courtyards of settlements of the South Kazakhstan, Zhambyl, and Kyzylorda regions were used.

Ticks were pre-homogenized in microtubes with a metal pestle, 400 µl of lysis buffer (from the «DNA-sorb-B» kit, InterLabService, Russia) were added to the tubes, carefully vortexed and incubated at room temperature for 12 hours. DNA was isolated according to the «DNA-sorb-B» kit protocol. DNA concentration was determined spectrophotometrically using a NanoDrop1000 spectrophotometer.

Specific primers were selected and tested using PrimerSelect (DNAStar), BioEdit, and PrimerBlast (NCBI) web resource. In this case, the main parameters were taken into account: close annealing temperature of the forward and reverse primers, the length of the primers from 18–25 bp, and the low probability of the formation of secondary structures.

Designed amplification conditions with selected primers Eno_T.anul_F-270 and Eno_T.anul_R-720: the reaction mixture contained 15 pmol forward and reverse primers, 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 0.08% (v/v) Nonidet P40, 2.5 mM $MgCl_2$, 200 nM each dNTP, 2 Unit Taq DNA polymerase (Alpha ferment, Russia); PCR was performed on a Mastercycler pro (Eppendorf) thermal cycler with simulated cycling conditions for the Mastercycler gradient, the program included a primary denaturation of 95°C for 3 minutes; 42 cycles with denaturation at 95°C for 30 seconds, annealing of primers at 60°C for 40 seconds, elongation at 72°C for 50 seconds, final elongation of 72°C – 5 minutes.

Optimization of PCR was performed by annealing temperature of the primers and the concentration of magnesium in the working mixture from 1.5 mM to 3 mM in increments of 0.5 mM. The reaction mixture included 15 pmol of forward and reverse primers, 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 0.08% (v/v) Nonidet P40, 200 nM of each dNTP, 2 Unit Taq DNA polymerase (Alpha-enzyme, Russia). SyberGreen I nunleic (Sigma, S9430) was used as an intercalating dye in a final dilution of 0.003 fold. The PCR program included primary denaturation at 95°C for 3 minutes; 42 cycles with denaturation at 95°C - 30 seconds, annealing of primers in a gradient from 56°C to 65°C - 30 seconds, elongation at 72°C - 45 seconds, taking into account the results at 78°C - 10 seconds. Amplification was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) thermal cycler.

The amplified target DNA fragments were analyzed on a 1.5% agarose gel in the presence of ethidium bromide. As the electrode buffer 1x TAE buffer was used. The results were documented using the Gel-Doc gel documentation system (Bio-Rad) with QuantityOne software (Bio-Rad). As a molecular weight marker «DNALadder 1kb» (Fermentas) was used.

Purification of PCR products from unbound primers and dNTPs was carried out by the enzymatic method using Exonuclease I (Fermentas) and alkaline phosphatase (Shrimp Alkaline Phosphatase, Fermentas). The sequencing reaction was carried out using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applide Biosystems) according to the manufacturer's instructions, followed by fragment separation on a 3730xl DNA Analyzer (Applide Biosystems). Nucleotide sequences were analyzed and combined into a common sequence in SeqMan software (DNAStar). After that, terminal fragments (nucleotide sequences of primers, fragments having a low quality index) were removed. To build a phylogenetic tree, MEGA 5.0 was used; sequence alignment was performed according to the ClustalW algorithm.

RESULTS

In the present study, the *ENO* gene, which is responsible for coding the enolase enzyme, was chosen as a diagnostic target. Apicomplexans are lack of aerobic metabolism system and, therefore, anaerobic glycolysis is the only source of energy generation [16]. Enolase (2-phospho-D-glycerate hydrolase) is an essential dimeric glycolytic enzyme that acts as a catalyst for the interconversion of 2-phosphoglyceric acid and phosphoenolpyruvate during glycolysis [17]. The nucleotide sequence of this gene is hypervariable and the identity among *Theileria* subspecies constitutes 76.5% [18]. A representative sample was generated from the NCBI database, which consisted of 17 *ENO gene* sequences of the order of *Piroplasmida*, of which 8 were *Theileria spp* and 9 were *Babesia spp*. Nucleotide sequence alignment was performed using BioEdit software. As a result of the analysis of aligned nucleotide sequences, the most conserved regions were selected in all representatives of *Theileria annulata* but variable in the remaining species. Primers Eno_T.anul_F-270 and Eno_T.anul_R-720 were selected for these sites. When tested in PrimerBlast software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), the primers were strictly specific to the target gene; the average calculated annealing temperature of the primers was 59°C and did not differ between the primers by more than 1°C. The size of the amplified region is 451 bp which allows verification of specific annealing by direct sequencing method.

To identify *Theileria annulata* DNA for subsequent optimization of conditions, PCR was performed with the calculated conditions and with DNA isolated from 18 ticks. As a result, a specific PCR product with an expected size of 451 bp was detected in 4 samples (figure 1).

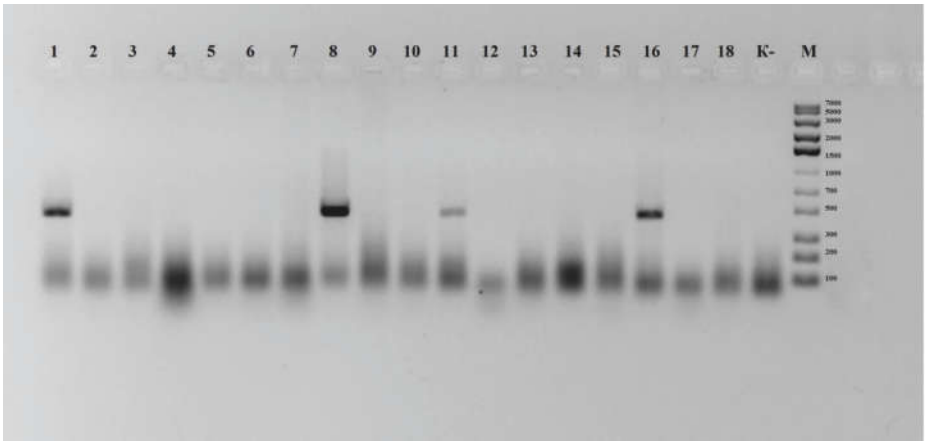

Fig. 1. The results of PCR analysis of DNA samples

PCR products were sequenced and the obtained nucleotide sequences were used for phylogenetic analysis with *enolase (ENO)* gene sequences from various species of the order *Piroplasmida* (figure 2).

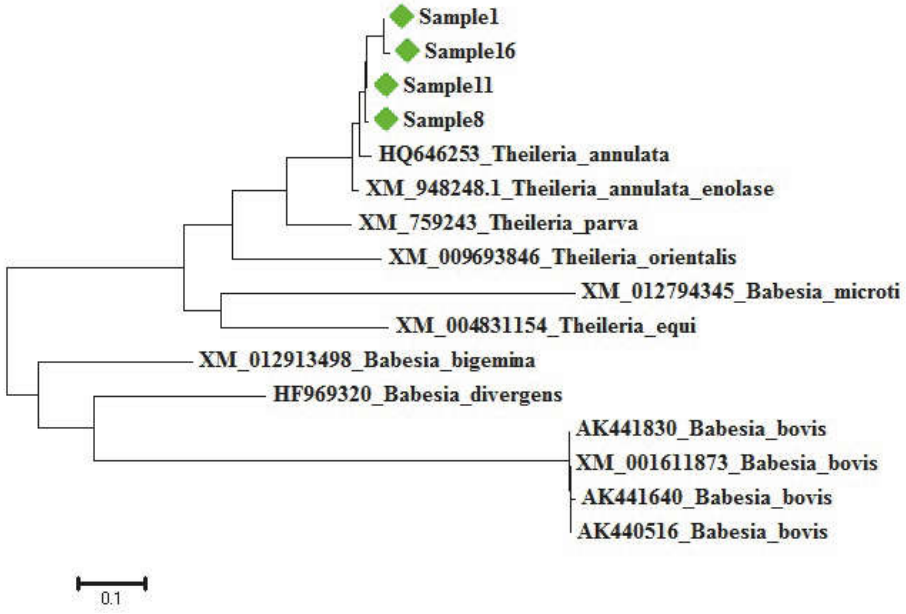

Fig. 2. Phylogenetic tree

As follows from the results of phylogenetic analysis, the sequence of samples 1, 8, 11, 16 clustered on the same branch with *Theileria annulata*. The data obtained confirm the specificity of the annealing of the used primers for the *Theileria annulata enolase (ENO)* gene. Tick DNA samples № 1, 16, 8, and 11 were combined into a pool for subsequent use in optimizing the conditions for PCR formulation.

The main variable component of the reaction mixture was the concentration of magnesium ion, which was determined in the range of 1.5-3.0 mM in increments of 0.5 mM at a temperature gradient from 56°C to 65°C. At a magnesium concentration of 1.5 mM and 2 mM, specific annealing was observed over the entire temperature gradient. With an increase in magnesium concentration to 2.5 mM - 3.0 mM, a weak yield of the PCR product was observed during electrophoretic analysis. At a magnesium concentration of 1.5 mM, in contrast to 2 mM, there were no significant differences in PCR efficiency over the entire temperature gradient, as evidenced by the range of CT (cycle threshold: +- 0.5 cycles from the average value. Nevertheless, the final fluorescence at the plateau level was different for the samples. The lowest level of fluorescence was observed in the wells with an annealing temperature of 56°C and 65°C. When electrophoretic accounting, it is seen that at low annealing temperatures (61.7-56°C), the formation of shimmers with a molecular weight of up to 200 bp is observed. Thus, the annealing temperatures of the primers 63.5°C and 64.5°C (on average 64°C) and the concentration of magnesium ions of 1.5 mM were the most optimal values for the pair of primers Eno_T.anul_F-270 and Eno_T.anul_R-720. The results are shown in figure 3.

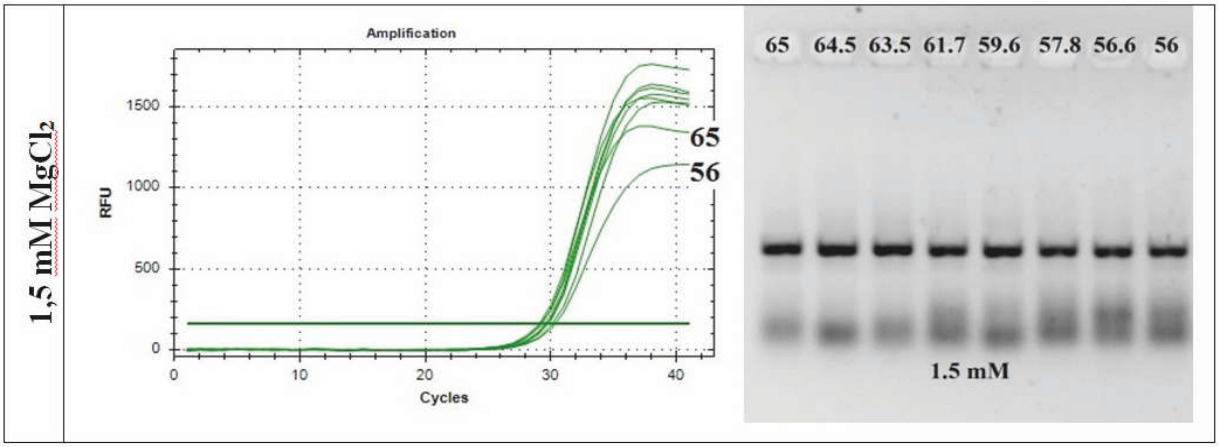

Fig. 3. Optimization of annealing temperature of the Eno_T.anul_F-270 and Eno_T.anul_R-720 primers and the concentration of magnesium ions in PCR mixture

Thus it was determined the final version of the PCR protocol: the reaction mixture contained 15 pmol forward and reverse primers, 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 0.08% (v/v) Nonidet P40, 1.5 mM $MgCl_2$, 200 nM each dNTP, 2 Unit Taq DNA polymerase; the program included a primary denaturation of 95°C for 3 minutes; 42 cycles with denaturation at 95°C for 30 seconds, annealing of primers at 64°C for 30 seconds, elongation at 72°C for 50 seconds, final elongation of 72°C – 5 minutes.

DISCUSSION AND CONCLUSION

As a result of this work, a PCR protocol was established to detect *Theileria annulata* DNA using the *enolase (ENO)* gene as a diagnostic target. By analyzing the nucleotide sequences of *Theileria spp*, the design of a pair of primers (Eno_T.anul_F-270 and Eno_T.anul_R-720) was established, which, when tested in the Primer Blast web resource, were strictly specific to the target gene. PCR analysis of the taken DNA samples of 18 ticks showed positive results in four DNA samples. Sequence of PCR products correspond to 97-100% of the species *Theileria annulata*. As a result of optimization of the PCR formulation protocol, it was found that the optimal annealing temperature is 64°C, with a concentration of magnesium ions of 1.5 mM.

The data obtained can be used in the development of methods for genetic diagnosis and prevention of bovine theileriosis.

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THEILERIA ANNULATA АНЫҚТАУҒА АРНАЛҒАН ПТР ХАТТАМАСЫН ОҢТАЙЛАНДЫРУ

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ТҮЙІН

Тейлериоз – аурудың ауырлық деңгейі әртүрлі, көп жағдайда малдың өлімін тудыратын үй және жабайы жануарлардың қанпаразитарлық ауруы. Бұл ауру Қазақстанның оңтүстік өңірлерінде эндемиялық сипатта болып табылады. Елімізде тейлериоз ауруымен қарсы күрестің негізгі іс-шараларына диагностика мен емдеу жатқызылады. Осыған орай, инфекцияны уақытында анықтау аурудың алдын алу шараларындағы негізгі құрал болып табылады. Полимеразды тізбекті реакция (ПТР) аурудың жасырын жүруін анықтауға мүмкіндік береді, сонымен қатар бұл әдіс классикалық микроскопиялық зерттеулермен салыстырғанда жоғары сезімталдыққа ие. Осы жұмыстың мақсаты диагностикалық нысан ретінде *enolase* (ENO) генін қолдану арқылы *Theileria annulata* ДНҚ-сын анықтау үшін ПТР анализінің оңтайлы параметрлерін анықтау болды. ПТР реакциясының есептік көрсеткіштері кезінде іріктелген праймерлер *Theileria annulata* қоздырғышын кенеден бөлінген 18 ДНҚ сынамаларының 4-інде анықтауға мүмкіндік берді. Келесі оңтайландыру шаралары ПТР тиімділігін арттырды. Әзірленген хаттаманың өзгешелігі оң сынамаларды тікелей секвенирлеу арқылы расталды.

Негізгі сөздер: тейлериоз, иксодты кене, ПТР, секвенирлеу, филогенетикалық талдау.

ОПТИМИЗАЦИЯ ПРОТОКОЛА ПЦР ДЛЯ ВЫЯВЛЕНИЯ *THEILERIA ANNULATA*

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АБСТАКТ

Тейлериоз – кровопаразитарное заболевание домашних и диких животных, с разной степенью тяжести течения, нередко приводящее к летальному исходу. Заболевание эндемично в южных регионах Казахстана. Основными средствами борьбы с кровопаразитарными инфекциями в Казахстане является диагностика и лечение. В связи с этим, своевременное выявление инфекции является критически важным этапом в процессе профилактических мероприятий. Полимеразная цепная реакция (ПЦР) позволяет выявлять скрытое носительство и обладает более высокой чувствительностью в сравнении с классическими микроскопическими исследованиями. Целью настоящей работы было определение оптимальных параметров постановки ПЦР для выявления ДНК *Theileria annulata*, с использованием в качестве диагностической мишени гена *enolase* (ENO). Подобранные праймеры при расчётных показателях ПЦР реакции позволили выявить *Theileria annulata* в 4 из 18 образцов ДНК, выделенных из клещей. Последующая оптимизация условий повысила эффективность ПЦР. Специфичность разработанного протокола подтверждена прямым секвенированием положительных образцов.

Ключевые слова: тейлериоз, иксодовый клещ, ПЦР, секвенирование, филогенетический анализ.