

Original papers

Identification of the piroplasms isolated from horses with clinical piroplasmosis in Poland

Łukasz Adaszek[1], Marta Górna[1], Michał Krzysiak[2], Michał Adaszek[3], Marcin Garbal[1], Stanisław Winiarczyk[1]

[1] Department of Epizootiology and Clinic of Infectious Diseases, Faculty of Veterinary Medicine, University of Life Sciences, 30 Gleboka Street, 20-612 Lublin, Poland
[2] Białowieza National Park, 11 Park Pałacowy Street, 17-230 Białowieza, Poland
[3] Czerniakowski Hospital S.P.Z.O.Z, 19/25 Stępińska Street, 00-739 Warsaw, Poland

Corresponding author: Łukasz Adaszek; E-mail: ukaszek0@wp.pl

ABSTRACT. The study was aimed at determining the cause of the diseases in three horses exhibiting symptoms of fever, ataxia, mucus membrane paleness, haematuria and thrombocytopenia. The PCR technique revealed the presence in the blood of 18S RNA *Babesia/Theileria* spp. genetic material. DNA amplification using primers RLB F2 and RLB R2 produced 430 bp size products. The sequences of these PCR products demonstrated a 95.6–97.5% similarity with the sequence of the fragment of 18S RNA *Babesia equi*, gene number DQ287951 in the GenBank. The treatment utilizing the subcutaneous application of the imidocarb resulted in gradual recovery of the diseased animals.

Key words: *Babesia* spp., *Theileria* spp., *Ehrlichia* spp., *Anaplasma* spp., rickettsiae, piroplasms, tick-borne diseases, horses, PCR

Introduction

Equine piroplasmosis is a serious disease caused by blood parasites such as *Theileria equi* (earlier referred to as *Babesia equi*) and *Babesia caballi* [1]. This disease appears endemically in tropical and subtropical regions and *Boophilus*, *Hyalomma*, *Dermacentor and Rhipicephalus* ticks are the disease vectors. *Babesia caballi* is a pear-shaped or amoeba-shaped protozoan, with size of 2.5–5 μm. In the erythrocytes the parasite usually appears in pairs; with their narrower ends pointing toward each other. *Theileria equi* is a very small, spherical parasite, up to 2 μm in size, occurring in erythrocytes and in the leukocytes [2,3].

Another feature differentiating *Babesia* from *Theileria* is the slightly different behaviour of these parasites in the host's organism. The first one attacks the red blood cells immediately after penetrating the animal or human body, whereas *Theileria* sporozoites during the initial invasion phase enter lymphocytes and macrophages, where they undergo schizogony and then the merozoites penetrate the erythrocytes, where they are further divided. Thus, the feature differentiating *Theileria* from *Babesia* piroplasms is the occurrence of schizogony in lymphocytes or in erythrocytes [3,4].

The symptoms of babesiosis/theileriosis varied – infected horses can suffer from jaundice, muscle weakness, haemoglobinuria and fever. *Babesia* and *Theileria* cause destruction of the erythrocytes through mechanical damage, which is clinically manifested by anaemia. It is also a result of a protozoa-induced humoral and cellular response in the host's body, leading to increased phagocytosis of both – parasite-infected and protozoa-free erythrocytes. Destruction of the red blood cells is intensified as a result of opsonisation of parasite-invaded erythrocytes with IgG antibodies and destabilisation of the cell membrane by factors released by the protozoa. Anaemia is accompanied by circulatory disorders, hypoxia, development of

Table 1. Results of haematological examination of the blood collected from horses during the acute phase of piroplasmosis

Parameter	RBC (10^6/mm^3)	WBC (10^3/mm^3)	HGB (g/dL)	HCT(%)	PLT (10^3/mm^3)
Stallion	5.23	3.6	7.2	23	84.0
Mare (4-yr old)	4.66	4.7	6.4	20	55.0
Mare (6-yr old)	4.10	4.5	6.8	19	62.0
Baseline	7.5–10	5.5–12	8–18	25–52	150–400

Explanations: RBC – red blood cells, WBC – white blood cells, HGB – haematoglobulin, HCT – haematocrit, PLT – platelets.

metabolic acidosis and impairment of many internal organs, especially the liver and kidneys, manifested by jaundice and hematuria [5]. The course of the disease is usually acute but it could be subacute and chronic [2,6].

Standard diagnosis of piroplasmosis in the areas of its enzootic occurrence is done based on medical history (i.e., the presence of ticks on horses), clinical symptoms such as fever, anaemia, jaundice and haemoglobinuria, and on microscopic examination of blood smear collected from diseased animals [7,8]. To diagnose babesiosis/theileriosis and to assess the epizootic situation, which includes detecting subclinical infections with this protozoa, molecular biology methods are frequently applied, mainly the polymerase chain reaction (PCR) and subsequent sequencing of the amplification products [5,9,10].

During the last three years we have diagnosed three cases of piroplasm infections in horses from eastern regions of Poland. Their full clinical description was presented in earlier reports [5,11]. As piroplasmosis in horses is rarely diagnosed in Poland and its etiological factors are not well known, the purpose of the study was to identify the microorganisms isolated from infected and symptomatic horses and to determine the parasite species.

Material and methods

The study included three horses from the Lublin region, Poland: a 2-yr old Deutsche Reit Ponny stallion, and two, 4-yr old and 6-yr old Malopolski mares. All the horses have had contact with ticks, followed by clinical signs manifested by: walking disorders, fever, apathy, weakness, pale mucous membranes. Blood was also observed in the urine of both mares. Blood was collected from these horses for haematological, biochemical and molecular tests for babesiosis and anaplasmosis/ehrlichiosis.

Haematological tests. Blood for haematological tests was sampled to the EDTA tubes, shaken and tested in the Exigo veterinary haematology analyser (Boule, Sweden) (Table 1).

Blood smear test. Blood smears were prepared on degreased microscope glass slides, stained using the Diff Quick method, air dried, and examined under 100× immersion oil objective of an Olympus CH 20 microscope.

Biochemical tests. Blood for biochemical tests was placed in tubes with coagulation accelerator, centrifuged, and the serum was tested in a biochemistry analyser BS-130 (Cormay, Poland). The parameters were: urea concentration in mg/dl, creatinine concentration in mg/dl, total bilirubin concentration in mg/dl, alanine aminotransferase (ALT) activity and asparagine aminotransferase

Table 2. Results of biochemical examination of the blood collected from horses during the acute phase of piroplasmosis

Parameter	Urea (mg/dl)	Creatinine (mg/dl)	Total bilirubine (mg/dl)	ALT (IU/l)	AST (IU/l)
Stallion	57	1.9	2.4	66	601
Mare (4-yr old)	76	2.1	1.9	81	597
Mare (6-yr old)	82	2.1	2.1	69	344
Baseline	25–45	1.2–1.9	0.8–1.5	3–25	205–555

Explanations: ALT – alanine transaminase, AST – aspartate aminotransferase, IU – international units.

(AST) activity expressed in international units (IU) (Table 2).

DNA isolation. The DNA for analysis was extracted from 100 µl of fresh blood using the DNA blood mini kit (DNA Gdańsk, Poland).

PCR. DNA blood samples were tested using PCR for the presence of genetic material of *Babesia* spp. protozoa and *Ehrlichia/Anaplasma* spp. rickettsiae.

The PCR for *Babesia/Theileria* was carried out using a pair of primers: RLB R2 (5'-CTAAGAATTTCACCTCTGACAGT-3') and RLB F2 (5'-GACACAGGGAGGTAGTGACAAG-3'), to amplify a DNA section of approximately 430 bp of the conserved 18S RNA gene fragment. PCR primers for *Ehrlichia/Anaplasma* spp.: EHR 521 (5'-TGTAGGCGGTTCGGTAAGTTAA AG-3') and EHR 747 (5'-GCACTCATCG TTTAC AGCGTG-3'), amplified the DNA section of the conserved 16S rRNA gene part of rickettsiae of the length of 247 bp.

PCR was carried out in a Biometra thermocycler. DNA of *Babesia* spp. isolated from the blood of a dog with babesiosis [5] and DNA of *Anaplasma phagocytophilum* from human blood (National Reference Center for Borreliae of Max von Pettenkofer Institute of Ludwig Maximilian University of Munich, Germany) served as positive controls, while DNA from the blood of a healthy horse served as a negative control.

PCR for *Babesia/Theileria* comprised 40 cycles, where the denaturation step was at 94°C for 35 s, the annealing of primers at 51°C for 35 s and the extension of strands for 36 s at 72°C [13].

The composition of the reaction mixture was prepared after the concentrations of its individual components (i.e., $MgCl_2$, dNTP, primers, and Taq Polymerase) have been optimized. The best results were obtained at primer concentration of 50 pM and the following concentrations of: dNTP – 200 µM, $MgCl_2$ – 3.0 µM and 2.5 U of Taq Polymerase.

PCR for *Ehrlichia* spp. comprised 35 cycles, where the denaturation step was at 94°C for 30 s, the annealing of primers at 56°C for 30 s and the strand extension at 72°C for 45 s [14].

Electrophoresis. PCR products were analysed using the electrophoresis method on 1% agarose gel, in 10 V/cm TBE buffer for 50 min. Once amplification products were stained with ethidium bromide, and their size with regard to the weight standard DNA ladder 100 bp (Gibco BRL) was determined.

Sequencing. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen), and sequenced in the Laboratory of DNA Sequencing and Oligonucleotide Synthesis of the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland. The sequencing results were analysed using the Lasergene DNA Star software. The sequences of isolates obtained in the present study were analysed and compared to the sequences of the 18S RNA gene of *Babesia caballi*, *Babesia equi* and *Theileria equi* available in the NCBI GeneBank at numbers: Z15104, AY534883, DQ287951, AY534882 using the Lasergene DNA Star software.

Results

Results of haematological and biochemical blood tests are presented in Table 1 and 2. Low erythrocyte haemoglobin concentrations, decreased haematocrit, thrombocytopeania and increased bilirubin level evidence that the studied animals suffered from haemolytic anaemia. Blood smears

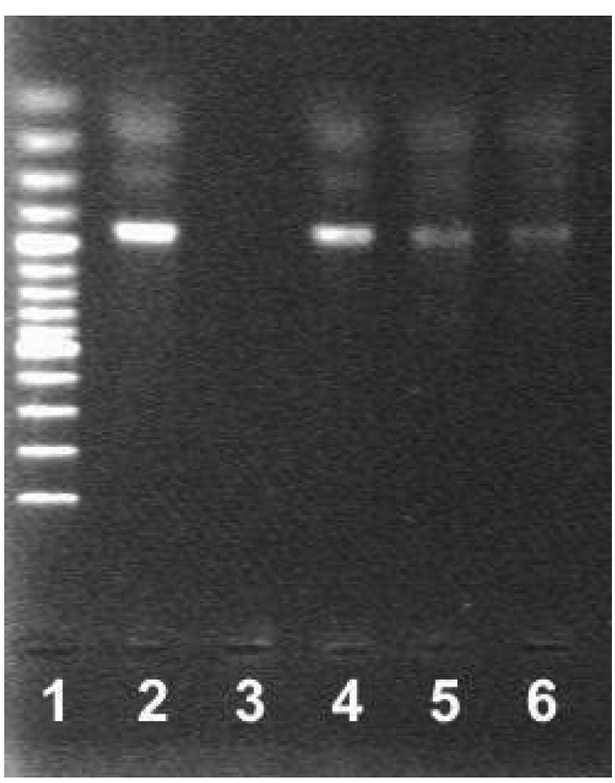

Fig. 1. Gel electrophoresis. PCR amplification of a partial sequence of 18S RNA *Babesia* spp./*Theileria* spp. gene, product size 430 bp. Lanes: (1) molecular weight marker = 100bp; (2) positive control; (3) negative control; (4-6) products of the amplified DNA isolated from blood of the diseased horses.

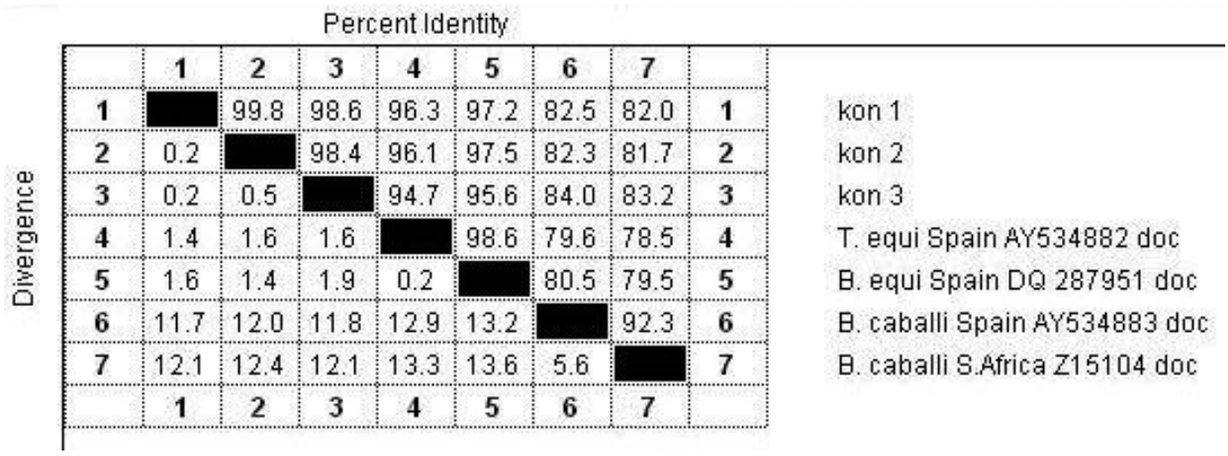

Fig. 2. Percent identity of a partial sequence of 18S RNA *Babesia* spp./*Theileria* spp. gene obtained in the present study sequences and the sequences deposited in the GenBank. Kon 1,2,3 = horse 1,2,3

did not reveal *Babesia* spp. protozoa in the erythrocytes or *Anaplasma/Ehrlichia* rickettsia in the leukocytes.

PCR test for *Babesia* spp. with RLB F2 and RLB R2 primers demonstrated the presence of genetic material of piroplasms of approximately 430 bp in the blood of all symptomatic horses (Fig. 1). In none of the tested blood samples was the *Ehrlichia/Anaplasma* genetic material detected.

The PCR product sequences developed using Lasergene DNA Star software showed a high similarity of 98.4–99.8%. Their comparison with standard sequences of the 18S RNA gene of *Babesia/Theileria* showed that the highest homology level (95.6–97.5%) occurred between isolates obtained in the present study and the *Babesia equi* DQ287951 sequence (Fig. 2). This is presented in the phylogenetic tree, where sequences of our isolates are situated on a separate branch near *B. equi* DQ287951 and *T. equi* AY534882 sequences, and are clearly far away from *Babesia caballi* sequences.

The causal treatment of horses using with Imizol (imidocarb dipropionate from Schering Plough Animal Health) at a dose of 2.0 ml/100 kg b.w. divided into two injections administrated every 12 hours) led to full recovery of the horses. Follow-up haematological tests performed a month after the treatment was commenced did not reveal any deviations from physiological baselines.

Discussion

This present study describes the cases of equine piroplasmosis, a disease that is rarely diagnosed in Poland. The fact that piroplasmosis regarded not so long ago as rather exotic disease, occurs now in Poland, evidences that pathogenic etiological factors are spreading in increasing numbers to the new areas. Such situation is without any doubt related to the increased animal trade and movement among various geographic regions. However,

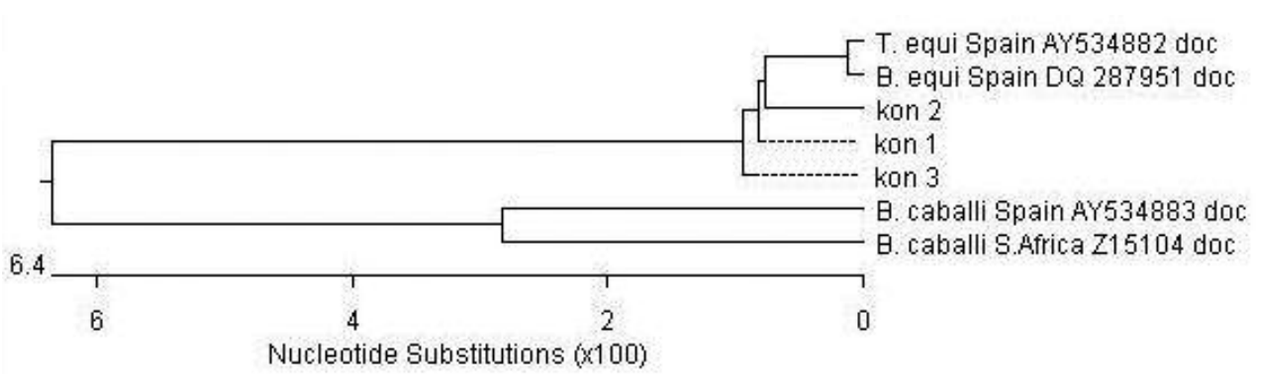

Fig. 3. Phylogenetic relationships among sequences of the isolates obtained in the present study and sequences deposited in the GenBank. Kon 1,2,3 = horse 1,2,3

climatic changes, manifested in climatic warming and thus creating favourable environmental conditions for disease transmission vectors in new areas, are also of significance.

Equine piroplasmosis is diagnosed relatively rarely. In Europe it was reported from Greece, France, Italy, Spain, Germany, UK and Turkey [15–21]. This disease can be fulminant with sudden death of the infected animals [22].

The results of the presented study demonstrated that the three horse cases disease were caused by infections with *Theileria equi*. The species of the isolated protozoa was determined based on PCR results and analysis of sequences of the amplicons. The high degree of similarity (i.e., 95.6–97.5%) of gene sequences between the protozoa isolated in the present study and the standard sequences from the gene bank confirmed the identification of this pathogen.

Also, haematological test results (i.e., thrombocytopenia and anaemia) and positive reaction of animals to imidocarb treatment point out the piroplasmosis.

The fact that molecular detection of the genetic material of protozoa in the blood of a horse with clinical signs was associated with a negative blood smear is not surprising. Contrary to canine *Babesia canis* parasites, equine *Theileria equi* parasites do not have characteristic pear-like shape. Furthermore, they are much smaller than the parasites attacking the red blood cells of dogs and they can be easily mistaken for particles characteristic for improperly blood smear stain. Also the degree of parasitaemia in *Theileria equi* and *Babesia caballi* infections seems to be much lower than parasitaemia observed in canine babesiosis. Due to all of the aforementioned factors, confirmation of the infection by the blood smear method for cases where equine piroplasmosis was detected molecularly, did not exceed 30% [6,23].

No effective immunoprophylaxis against equine piroplasmosis has been developed so far. Disease prevention involves preventing the protozoa-infected horses entering the areas free of these parasitic protozoa invasion. Horses transported from areas where babesiosis/theileriosis occurs ought to be tested upon arrival. Anti-tick prophylaxis is also an important element in fighting this disease [8].

The fact that equine infections with *Babesia/Theileria* have been identified in the present study demonstrates that domestic horses are at risk of contracting piroplasmosis. These infections must be considered every time when a contact between horses and ticks is followed by severe pathological symptoms accompanied by anaemia and thrombocytopenia revealed by haematological tests.

References

[1] Mehlhorn H., Schein E. 1998. Redescription of *Babesia equi* Laveran, 1901 as *Theileria equi* Mehlhorn, Schein 1998. *Parasitology Research* 84: 467-475.

[2] Gundłach L.J., Sadzikowski A.B., Studzińska M.B. 2006. Inwazje pierwotniaków u koni. *Annales UMCS, Sectio DD* 61: 31-44.

[3] Uilenberg G. 2006. *Babesia* – a historical overview. *Veterinary Parasitology* 138: 3-10.

[4] Adaszek Ł., Winiarczyk S., Górna M. 2010. Od piroplazmozy do babeszjozy – problemy w klasyfikacji pierwotniaków z rodzaju *Babesia. Wiadomości Parazytologiczne* 56: 111-115.

[5] Adaszek Ł. Winiarczyk S. 2008. Przypadek babeszjozy konia. *Medycyna Weterynaryjna* 64: 1317-1320.

[6] Rampersad J., Cesar E., Campbell M.D., Samlal M., Ammons D. 2003. A field evaluation of PCR for the routine detection of *Babesia equi* in horses. *Veterinary Parasitology* 114: 81-87.

[7] Battsetseg B., Lucero S., Xuan X., Claveria F.G., Inoue N., Alhassan A., Kanno T., Igarashi I., Nagasawa H., Mikami T., Fujisaki K. 2002. Detection of natural infection of *Boophilus microplus* with *Babesia equi* and *Babesia caballi* in Brazilian horses using nested polymerase chain reaction. *Veterinary Parasitology* 107: 351-357.

[8] Rashid A., Mubarak A., Hussain A. 2009. Babesiosis in equines in Pakistan: a clinical report. *Veterinaria Italiana* 45: 391-395.

[9] Criado A., Martinez J., Buling A., Barba J.C., Merino S., Jefferies R., Irwin P.J 2006. New data on epizootiology and genetics of piroplasms based on sequences of small ribosomal subunit and cytochrome b genes. *Veterinary Parasitology* 142: 238-247.

[10] Alhassan A., Govind Y., Tam N.T., Thekisoe O.M., Yokoyama N., Inoue N., Igarashi I. 2007. Comparative evaluation of the sensitivity of LAMP, PCR and in vitro culture methods for the diagnosis of equine piroplasmosis. *Parasitology Research* 100: 1165-1168.

[11] Adaszek Ł., Kotowicz W., Winiarczyk S. 2010. Przypadki piroplazmozy u koni w Polsce. *Weterynaria w Praktyce* 10: 90-92.

[12] Štefančíková A., Adaszek Ł., Peťko B., Winiarczyk S., Dudiňák V. 2008. Serological and immunochemical evidence of *Borrelia burgdorferi sensu lato* in horses and cattle from Poland and diagnostic problems of Lyme borreliosis. *Annals of*

Agricultural and Environmental Medicine 15: 37-43.
[13] Altay K., Aydin M.F., Dumanli N., Aktas M. 2008. Molecular detection of *Theileria* and *Babesia* infections in cattle. *Veterinary Parasitology* 158: 295-301.
[14] Adaszek Ł., Winiarczyk S., Łukaszewska J. 2009. A first case of ehrlichiosis in a horse in Poland. *Deutsche Tierärztliche Wochenschrift* 9: 330-334.
[15] Kouam M.K., Kantzoura V., Gajadhar A.A., Theis J.H., Papadopoulos E., Theodoropoulos G. 2010. Seroprevalence of equine piroplasms and host-related factors associated with infection in Greece. *Veterinary Parasitology* 169: 273-278.
[16] Fritz D. 2010. A PCR study of piroplasms in 166 dogs and 111 horses in France (March 2006 to March 2008). *Parasitology Research* 106: 1339-1342.
[17] Moretti A., Mangili V., Salvatori R., Maresca C., Scoccia E., Torina A., Moretta I., Gabrielli S., Tampieri M.P., Pietrobelli M. 2010. Prevalence and diagnosis of *Babesia* and *Theileria* infections in horses in Italy: a preliminary study. *Veterinary Journal* 184: 346-350.
[18] Camacho A.T., Guitian F.J., Pallas E., Gestal J.J., Olmeda A.S., Habela M.A., Telford S.R., Spielman A. 2005. *Theileria (Babesia) equi* and *Babesia caballi* infections in horses in Galicia, Spain. *Tropical Animal Health and Production* 37: 293-302.
[19] Boch J. 1985. *Babesia* infections in horses, cattle and dogs in southern Germany. *Tierarztliche Praxis* 1: 3-7.
[20] Joyner L.P., Donnelly J., Huck R.A. 1981. Complement fixation tests for equine piroplasmosis (*Babesia equi* and *B. caballi*) performed in the UK during 1976 to 1979. *Equine Veterinary Journal* 13: 103-106.
[21] Sevinc F., Maden M., Kumas C., Sevinc M., Ekici O.D. 2008. A comparative study on the prevalence of *Theileria equi* and *Babesia caballi* infections in horse sub-populations in Turkey. *Veterinary Parasitology* 156: 173-177.
[22] Vial H.J., Gorenflot A. 2006. Chemotherapy against babesiosis. *Veterinary Parasitology* 138: 147-160.
[23] Heim A., Passos L.M., Ribeiro M.F., Costa-Júnior L.M., Bastos C.V., Cabral D.D., Hirzmann J., Pfister K. 2007. Detection and molecular characterization of *Babesia caballi* and *Theileria equi* isolates from endemic areas of Brazil. *Parasitology Research* 102: 63-68.

Identyfikacja piroplazm izolowanych od koni w Polsce w klinicznych przypadkach choroby

Ł. Adaszek, M. Górna, M. Krzysiak, M. Adaszek, M. Garbal, S. Winiarczyk

Celem badań było określenie przyczyny choroby u trzech koni zdradzających objawy gorączki, niezborności ruchowej, bladości błon śluzowych, krwiomoczu, u których w badaniu hematologicznym wykazano trombocytopenię. Techniką PCR we krwi chorych zwierząt wykazano obecność materiału genetycznego 18S RNA *Babesia/Theileria* spp. W reakcji amplifikacji z wykorzystaniem starterów RLB F2 i RLB R2 uzyskano produkt o wielkości 430 pz, którego sekwencja nukleotydowa wykazywała 95,6–97,2% podobieństwo z sekwencją fragmentu genu 18S RNA *Babesia equi*, umieszczoną w banku genów pod numerem DQ28795. W leczeniu chorych zwierząt zastosowano imidokarb podawany podskórnie. W następstwie podjętej terapii obserwowano stopniowy powrót koni do zdrowia.

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