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MOLECULAR, PHYLOGENETIC AND HEMATOLOGICAL ANALYSIS OF A THEILERIA EQUI ISOLATE: A CLINICAL CASE REPORT

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Abstract: *Equine theileriosis is a tick borne haemoprotozoan disease caused by Apicomplexa protozoan Theileria equi, a piroplasm transmitted by Dermacentor spp., Hyaloma spp. and Rhipicephalus spp. tick species. The disease is associated with the presence of tick vectors in the area and long-term carrier horses. The present case report involves a 2-year-old mare presenting mild fever and periods of lethargy, decreased appetite and an increased tendency to remain in decubitus. Hematological report showed microcytic anemia, lymphocytosis and monocytosis. Molecular analysis revealed the presence of Theileria equi and phylogenetic analysis revealed close genetical links with isolates deposited in GenBank. The study provides information to practicing veterinarians about a disease with serious manifestations and confirms the continued expansion of the pathogen in new territories*

• Introduction

Equine piroplasmosis (EP) is a tick-borne disease of equids caused by the hemoprotozoan parasites *Theileria equi* and *Babesia caballi*. Among these, *T. equi* is particularly significant due to its wide geographic distribution, capacity to establish long-term infections, and potential to cause severe, sometimes fatal, clinical manifestations. The disease is transmitted by Ixodid ticks and is characterized by fever, anemia, icterus, and, in severe cases, hemoglobinuria and death. In endemic regions, EP poses a considerable threat to equine health and productivity, and it is a major constraint on international horse movement due to stringent disease-free certification requirements. Although *T. equi* has been reported in many parts of the world, including Europe, data on its molecular characterization and phylogenetic relationships in Eastern European countries, such as Romania, remain limited. Furthermore, few studies have comprehensively linked molecular findings with clinical and hematological profiles in naturally infected horses. This study presents the first combined molecular, phylogenetic, and hematological investigation of a *Theileria equi* isolate from a clinically affected horse in Romania. By integrating clinical observations with genetic characterization, this case report aims to contribute to the growing body of knowledge on *T. equi* diversity and pathogenesis, while also providing insights into its potential epidemiological implications for the Romanian equine population.

• Material and method

The present case report involves a 2-year-old mare presenting at Clinics of the Faculty of Veterinary Medicine Timișoara. The horse was brought to the veterinary clinic due to a progressively deteriorating general condition, with initial suspicion of tetanus expressed by the owner. The clinical signs started insidiously, within approximately 7-10 days, with periods of lethargy, decreased appetite and an increased tendency to remain recumbent. In the last few days, the animal showed marked difficulty in getting up from the ground, intermittent muscle fasciculations, reduced water consumption and marked contracture of the flexor muscles of the hind limbs. The owner mentioned that the animal had been exposed during the summer in an area endemic for vector-borne diseases without regular prophylactic parasite treatments.

On clinical examination, the horse had a slightly elevated heart rate (approximately 50 beats/min) and respiratory rate within physiological limits. The apparent mucous membranes were slightly anaemic and mildly subicteric, indicating possible haematological and hepatobiliary disorders. Capillary refilling time was prolonged (~3 seconds) and body temperature was slightly elevated (38.9°C). The general condition was apathetic, with obvious impairment of body condition and a depressed attitude. There was hepatomegaly on deep palpation and discrete tenderness in the lumbar area. Examination of the urine revealed a darker than normal colouration, possibly suggestive of haemoglobinuria or bilirubinuria. Neurological signs were not obviously present, but mild incoordination on gait was noted.

The sample of blood were subjected to stained smear examination and molecular analysis for the identification of Piroplasm DNA using the ISOLATE II Genomic DNA Kit (Meridian Bioscience, London, UK). The 18S rRNA gene in the mitochondrial genome of Babesia/Theileria spp. was amplified by polymerase chain reaction (PCR) using Primers: BJ1: 5'-GTCTTGTAATTGGAATGATGG-3' and BN2 5' – TAGTTTATGGTTAGGACTACG-3' described by Casati et al. (2006). A MyTaqTM Red Mix Master Mix (Meridian Bioscience, London, UK). was used for the reaction. The amplification program was carried out with the My Cycler thermocycler (BioRad®). This program included the steps of DNA denaturation at 95°C for 1 minute; 32 cycles of: denaturation at 95°C for 30 seconds, hybridization at 55°C for 30 seconds and extension at 72°C for 30 seconds; followed by incubation at 4°C. Amplicon analysis and control was performed by horizontal electrophoresis in a 1.5% agarose gel and images were captured with a digital camera.

A part of the positive samples were sequenced by the biotechnology company Macrogene®. The sequences (Fig 1) were edited and subsequently identified and compared with the sequences of both haplotypes present in Genbank® using the BLAST® tool. Phylogenetic analyses were performed using Phylogeny.fr (http://www.phylogeny.fr/)

• Results and discussions

Examination of stained smears did not identificate piroplasms in the erythrocytes or lymphocytes.

Hematological report showed microcytic anemia, lymphocytosis and monocytosis.

In the Biochemical analysis a increase in total bilirubin was identificated.

Molecular analysis testing the 18S rRNA gene succesfuly amplificated ~450 bp revealing the presence of *Theileria equi* DNA at. (Fig. 1)

BLAST analysis revealed 100% similarities with isolates MT093500.1 (from China), LC431546.1 (From Japan), MF510476.1 (from France) deposited in GenBank (Fig 2, 3).

The sequence was deposited in GenBank with accession number **PV611742** (Fig. 4).

At the evaluation of the results of phylogeny (Fig. 4). The Romanian isolate clusters with a group of other *Theileria equi* sequences genotype D, part of the same group or clade. This grouping suggests that the Romanian isolate is closely related to other *T. equi* strains in this part of the tree. Bootstrap value indicate the confidence in branching point as values close to 1.0 (or 100%) mean very high confidence. The node connecting the Romanian isolate has a bootstrap value of 0.994, indicating strong support for this evolutionary relationship. Comparation with isolates from the same clade included our isoalate in the Genotype D.

Theileria equi has been categorized until recent studies into three genetic groups, labeled A, B, and C. Up until now, no changes have been made to the genetic classification of *B. caballi*, aside from splitting group A into A1 and A2. Research by Salim et al. 2010 and Hall et al. 2013 identified four genetic groups for *T. equi*. Later studies by Qablan et al. 2013, Liu et al. 2016 and Facile et al. 2025 further expanded this classification to include five groups, named A through E. Nevertheless, this categorization has yet to gain universal acceptance in all phylogenetic research concerning these piroplasms. The inconsistencies present in both the data and the sequences documented in the literature complicate the establishment of a unified naming system.

Until now three investigations have examined the prevalence among domestic horses in wastern and central Romania (Giubega et al. 2022, Muresan et al 2023) and semi-feral horses located in the Danube Delta (Gallusová et al. 2014) proving the presence in the area of piroplasm in horses. Molecular evidence and also the GenBank deposit of the isolates enriches the knowledge of this disease in Romania.

• Conclusions

Although microscopic examination of blood smears did not reveal visible piroplasms, hematological and biochemical alterations—namely microcytic anemia, lymphocytosis, monocytosis, and elevated total bilirubin—suggested a possible hemoparasitic infection. Molecular analyses revealed the presence of the pathogen.

The Romanian *Theileria equi* isolate is part of a well-supported clade of *T. equi.*

The phylogenetic analysis reveals that the *Theileria equi* isolate from Romania (accession PV611742.1) clusters closely with other *T. equi* strains, forming a well-supported clade with high bootstrap values (up to 0.994). This indicates a strong evolutionary relationship and suggests that the Romanian isolate shares a recent common ancestor with these strains.

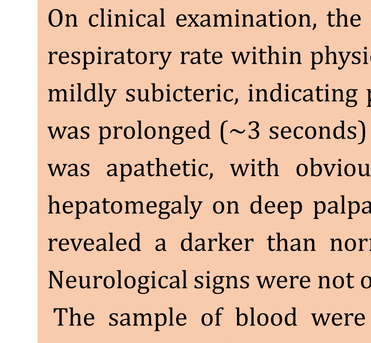

Fig. 1 Gel electrophoresis image showing positive results for *Babesia/Theileria* spp.

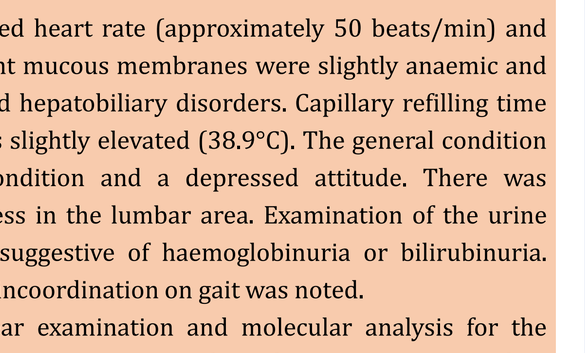

Fig. 2, 3 Sequencing confirmation of *Theileria* spp.

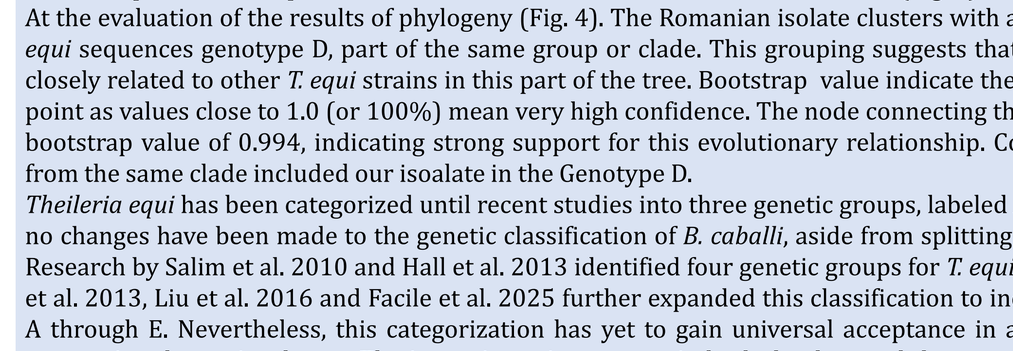

Fig. 4. Phylogenetic tree constructed using the Neighbor-Joining method revealing the close relation of our isolate with other *T. equi* isolates

Fig. 4 Deposited GenBank isolate

BLASTN 2.8.0+

Reference:
Zheng Zhang, Scott Schwartz, Lukas Wagner, and Webb Miller (2000), "A greedy algorithm for aligning DNA sequences", J Comput Biol 2000; 7(1-2):203-14.

Database: Nucleotide collection (nt)
89,336,887 sequences; 1,101,859,236,486 total letters

Query= E241126-003_I05_628_P5 1 1377

Length=1377

Sequences producing significant alignments:		Score (Bits)	E Value
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MT093493.1	Theileria equi isolate JL04 small subunit ribosomal R...	750	0.0
MT093471.1	Theileria equi isolate JL02 small subunit ribosomal R...	750	0.0
MN857679.1	Theileria equi isolate M8 small subunit ribosomal RNA...	750	0.0
MN857677.1	Theileria equi isolate M6 small subunit ribosomal RNA...	750	0.0
LC431546.1	Theileria equi KSA_D_Th2 gene for 18S ribosomal RNA, ...	750	0.0
MH651217.1	Theileria equi clone c371 18S ribosomal RNA gene, par...	750	0.0
MH651213.1	Theileria equi clone c38 18S ribosomal RNA gene, part...	750	0.0
MH651212.1	Theileria equi clone c72 18S ribosomal RNA gene, part...	750	0.0
MF510476.1	Theileria sp. Europa voucher marcv02 18S ribosomal RN...	750	0.0
MG551915.1	Theileria equi isolate RUS/Nov-h7 small subunit ribos...	750	0.0

GenBank

Theileria equi isolate RO_628 small subunit ribosomal RNA gene, partial sequence

GenBank: PV611742.1
FASTA Graphics

Go to:

LOCUS PV611742 410 bp DNA linear INV 12-MAY-2025
DEFINITION Theileria equi isolate RO_628 small subunit ribosomal RNA gene, partial sequence.
ACCESSION PV611742
VERSION PV611742.1
KEYWORDS .
SOURCE Theileria equi
ORGANISM Theileria equi
Eukaryota; Sar; Alveolata; Apicomplexa; Aconoidasida; Piroplasmida; Theileriidae; Theileria.
REFERENCE 1 (bases 1 to 410)
AUTHORS Imre,M., Pocinoc,A., Nistor,P., Dumitru,S., Ilie,M. and Morariu,S.
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COMMENT ##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##
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