Clinico-Biochemical and Molecular Analysis of *Theileria equi* and *Anaplasma phagocytophilum* Co-Infection in a Thoroughbred Mare in Aq-Qala, Iran

Amirali Avarseji[1]*, Farima Jahromi[2], Saeed Kashi[3], Soheil Mohammadparast[4]

1- Department of Internal Medicine, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
2- Department of Clinical Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran
3- Department of Veterinary Medicine, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
4- Department of Veterinary Medicine, Faculty of Veterinary Medicine, Islamic Azad University, Tabriz Branch, Tabriz, Iran.

Corresponding author: amirali.avarseji@ut.ac.ir

Abstract

Tick-borne diseases like *Theileria equi* and *Anaplasma phagocytophilum* pose a major threat to horses. Although both are present in Iran, confirmed co-infections are rarely documented, even though they can synergistically complicate clinical diagnosis. This report describes a rare, molecularly confirmed co-infection in a 4-year-old Thoroughbred mare from Aq-Qala, Golestan province, Northern Iran. The mare had a history of tick exposure in an endemic area during peak tick activity. Clinically, she presented with acute fever (39.3°C), tachycardia (45 beats/min), tachypnea (26 breaths/min), and pale mucous membranes, indicating hemolytic compromise. Despite clinical signs of anemia, automated hematological analysis revealed severe hemoconcentration (hematocrit: 63.4%; red blood cell count: 14.8×10^6/µL) — a "masked anemia" phenomenon where dehydration and splenic contraction obscure the underlying hemolytic state — along with marked leukocytosis (23.6×10^3/µL) with a regenerative left shift (4% band neutrophils). Serum biochemical profiling further demonstrated severe hypoglycemia (33 mg/dl), hyperbilirubinemia (5.70 mg/dl), and significantly elevated activities of lactate dehydrogenase (953 U/l) and gamma-glutamyl transferase (186 U/l). Definitive diagnosis was achieved through conventional polymerase chain reaction (PCR) targeting the 18S and 16S rRNA genes, confirming simultaneous infection with *T. equi* and *A. phagocytophilum*. These findings highlight a "masked anemia" phenomenon, where severe dehydration and splenic contraction in response to acute infection and stress artificially inflated the circulating red cell mass, obscuring the underlying hemolytic stateThis case

shows that in febrile horses from endemic areas, automated hematology alone is insufficient, and clinical examination should be supported by molecular diagnostics for accurate detection of co-infections.

Keywords: *Anaplasma phagocytophilum*, Equine, Iran, Mare, Polymerase Chain Reaction, *Theileria equi*.

1. Introduction

Tick-borne diseases (TBDs) represent a significant health and economic challenge in global equine populations, particularly in regions where vector ticks are endemic [1]. These infections can lead to substantial economic losses characterized by decreased athletic performance, trade restrictions, and increased mortality rates. Among the most clinically important equine tick-borne pathogens are *Theileria equi*, the primary cause of equine piroplasmosis (EP), and *Anaplasma phagocytophilum*, the etiological agent of equine granulocytic anaplasmosis (EGA) [2,3].

While both pathogens are known to circulate in various provinces of Iran, particularly in the northern regions where environmental conditions favor vector tick populations [4,5], molecularly confirmed reports of their simultaneous infection remain notably limited. Co-infections are particularly challenging in veterinary medicine as they can synergistically alter disease severity and modify typical laboratory markers, potentially leading to significant diagnostic pitfalls [6]. Therefore, the present report describes the molecular detection of a naturally occurring co-infection of *T. equi* and *A. phagocytophilum* in a Thoroughbred mare from the Aq-Qala region, Northern Iran. This study aims to analyze the associated clinico-pathological alterations and discuss the implications of multi-pathogen infections for clinical diagnosis in endemic areas.

2. Case Presentation

In November 2025, a 4-year-old Thoroughbred mare from the Aq-Qala region, Northern Iran, was presented for clinical evaluation due to acute onset of lethargy and anorexia. Clinical examination revealed pyrexia (39.3˚C), tachycardia (45 beats/min), and tachypnea (26 breaths/min). Physical findings included pale mucous membranes and a prolonged capillary refill time (>2 s), suggesting severe dehydration and potential hemolytic compromise.

2.1. Hematological and Biochemical Evaluation

The automated hematological analysis and serum biochemical profiling revealed significant alterations, as summarized in Table 1. Automated hematology revealed severe hemoconcentration, with a hematocrit (HCT) of 63.4% and a red blood cell (RBC) count of 14.8×10^6/µL, which significantly exceeded standard reference

ranges [7] and masked the underlying anemia. A significant leukocytosis ($23.6\times10^3/\mu L$) was recorded. Manual differential count showed band neutrophils, indicating a regenerative left shift. Serum biochemistry demonstrated marked hypoglycemia (33 mg/dl), hyperbilirubinemia (5.70 mg/dl), and significantly elevated activities of lactate dehydrogenase (LDH: 953 U/l) and gamma-glutamyl transferase (GGT: 186 U/l). Additionally, hypercalcemia (14.6 mg/dl) and hypomagnesemia (1.90 mg/dl) were observed.

Table 1. Hematological and Serum Biochemical Findings in a Thoroughbred Mare Co-infected with *Theileria equi* and *Anaplasma phagocytophilum*.

Parameter	Unit	Patient Value	Reference Range (Equine)
Hematology (CBC)			
White Blood Cell (WBC)	$\times10^3/\mu L$	23.6	5.4−14.3
Red Blood Cell (RBC)	$\times10^6/\mu L$	14.8	6.8−12.9
Hemoglobin (HGB)	g/dL	21.9	11.0−19.0
Hematocrit (HCT)	%	63.4	32.0−53.0
Mean Corpuscular Volume (MCV)	fL	42.8	37.0−58.5
Platelet (PLT)	$\times10^3/\mu L$	209	117−446
Differential Count			
Band Neutrophils	%	4	0−2
Segmented Neutrophils	%	40	30−75
Lymphocytes	%	52	20−70
Serum Biochemistry			
Glucose	mg/dl	33	75−115

Total Bilirubin	mg/dl	5.7	0.5−2.3
Gamma-Glutamyl Transferase (GGT)	U/l	186	5−24
Lactate Dehydrogenase (LDH)	U/l	953	162−412
Total Protein	g/dl	8.6	5.2−7.9
Total Cholesterol	mg/dl	268	75−150
Triglycerides	mg/dl	78	10−70
Calcium	mg/dl	14.6	10.2−13.4
Magnesium	mg/dl	1.9	2.2−2.8

2.2. Molecular Diagnosis

Whole blood samples were collected in EDTA tubes. Genomic DNA was extracted from 200 µL of whole blood using a commercial genomic DNA extraction kit according to the manufacturer's instructions. Extracted DNA was stored at −20 ˚C until further analysis.

2.2.1. Detection of *Theileria equi*

Conventional PCR targeting the 18S rRNA gene of *Theileria equi* was performed as previously described [8,9,11]. Forward and reverse primers targeting a conserved region of the 18S rRNA gene were used to amplify an approximately 260 bp fragment. The 25 µL reaction mixture contained 12.5 µL of 2× PCR Master Mix, 1 µL of each primer (10 pmol/µL), 2 µL of template DNA, and 8.5 µL nuclease-free water. Thermal cycling conditions included initial denaturation at 95 ˚C for 10 min, followed by 35 cycles of denaturation at 94 ˚C (30 s), annealing at 60 ˚C (45 s), and extension at 72 ˚C (45 s), with a final extension at 72 ˚C for 5 min. The PCR products were visualized on a 2% agarose gel, revealing a specific band at 260 bp for *T. equi* (Figure 1).

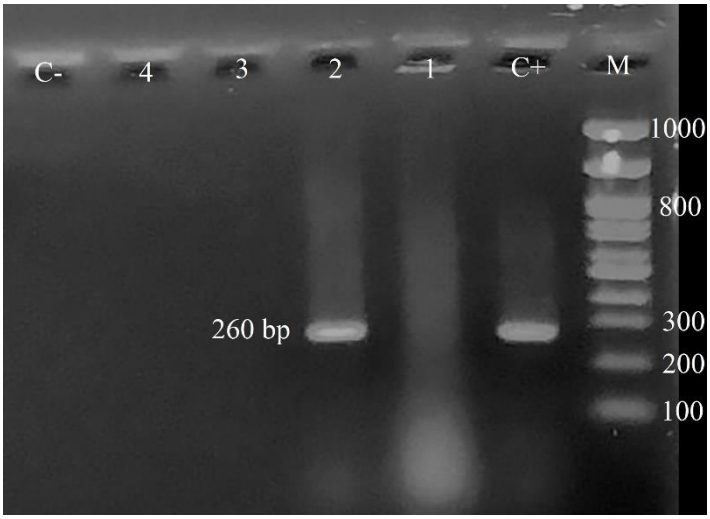

Figure 1. Agarose gel electrophoresis (2%) of PCR products amplified from the 18S rRNA gene of *Theileria equi*. Lane M: DNA ladder; Lane C+: Positive control; Lane 1: Negative sample; Lane 2: Positive sample from the Thoroughbred mare showing a specific band at 260 bp; Lanes 3-4: Other equine samples; Lane C-: Negative control.

2.2.2. Detection of *Anaplasma phagocytophilum*

PCR amplification of the 16S rRNA gene of *Anaplasma phagocytophilum* was performed based on established protocols [4,10]. Primer pairs designed to amplify an approximately 919 bp fragment were utilized. The reaction was carried out in a final volume of 25 µL containing 12.5 µL of 2× PCR Master Mix, 1 µL of each primer (10 pmol/µL), 2 µL of template DNA, and 8.5 µL nuclease-free water. The thermal profile consisted of initial denaturation at 95 ˚C for 5 min, 35 cycles of 94 ˚C (30 s), 58 ˚C (45 s), and 72 ˚C (1 min), followed by a final extension at 72 ˚C for 7 min. PCR products were analyzed via electrophoresis on a 2% agarose gel stained with ethidium bromide. Similarly, the amplification of the 16S rRNA gene confirmed the presence of *A. phagocytophilum* with a distinct band at 919 bp (Figure 2).

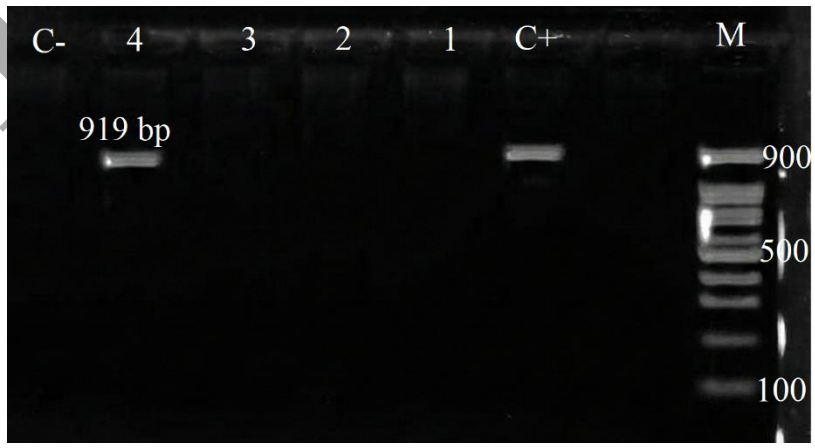

Figure 2. Agarose gel electrophoresis (2%) of PCR products amplified from the 16S rRNA gene of *Anaplasma phagocytophilum*. Lane M: DNA ladder; Lane C+: Positive control; Lanes 1-3: Negative samples; Lane 4: Positive sample from the Thoroughbred mare showing a specific amplicon at 919 bp; Lane C-: Negative control.

3. Discussion

The simultaneous detection of *T. equi* and *A. phagocytophilum* in Northern Iran aligns with regional surveys identifying these pathogens as endemic in the Caspian littoral [4,5]. A critical diagnostic challenge in this case was the "masked anemia"; despite clinical evidence of hemolysis — confirmed by elevated LDH (953 U/l) and total bilirubin (5.70 mg/dl) — the 63.4% hematocrit and 14.8×10^6/µL RBC count resulted from severe dehydration and splenic contraction in response to acute pyrexia (39.3˚C) and stress [2,6,7]. Recent molecular surveillance in neighboring regions, such as Türkiye and Eastern Europe, has similarly identified a high prevalence of concurrent tick-borne infections, emphasizing the regional epidemiological risk [12,13].

The synergy between these pathogens likely exacerbated the systemic inflammatory response and metabolic demand, leading to the observed hypoglycemia (33 mg/dl) and hepatic stress. In conclusion, this case underscores that clinicians should not rely solely on automated hematology in febrile equids in endemic areas, as physiological responses can mask underlying pathological states. Integrating detailed clinical evaluation with molecular tools such as PCR is indispensable for accurate diagnosis and effective monitoring of complex multi-pathogen infections.

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Authors' Contributions:

Amirali Avarseji and Saeed Kashi conceived and designed the study. Farima Jahromi performed the clinical examination and sample collection. A.A and S.K carried out the molecular analyses. F.J and Soheil Mohammd Parsat analyzed and interpreted the data .A.A drafted the manuscript. All authors reviewed and approved the final manuscript.

Ethics: All principles of medical ethics have been observed in this study.

Conflict of Interest: The authors declare no competing interests.

Data Availability: All data supporting the findings of this study are available within the article.

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