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Original Article

Molecular detection and genotyping of *Theileria equi* infection within the equine population in Giza, Egypt, using real-time PCR as compared with conventional detection methods

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Abstract

Background: Equine piroplasmosis represents one of the main and serious health problems affecting the equines industry globally, caused by a tick-borne protozoa called *Theileria equi* and *Babesia caballi*. **Aims:** This study aimed to identify and genotype *T. equi* within the equine population from Giza Governorate, Egypt, by comparing the obtained results using the available diagnostic methods. **Methods:** We collected 116 apparently healthy horses from the study area during the first half of 2019 to identify *T. equi* using real-time PCR (qPCR), targeting the *18S rRNA* gene. The results were compared with those from microscopic examination of Giemsa-stained blood smears and conventional PCR. Genotyping of the obtained sequences was also conducted to explore the genetic diversity of the detected *T. equi* strains. **Results:** Through sequencing and phylogenetic analysis, our samples were grouped into clusters corresponding to genotype A and genotype E. Our results demonstrated that the qPCR had the highest sensitivity (100%) followed by conventional PCR (68%) while microscopic examination had the lowest sensitivity (38%). Furthermore, the negative predictive value (NPV) of qPCR was the highest (100%) compared with conventional PCR and microscopical examination (80.49% and 68.04%, respectively) which revealed that negative cases detected by qPCR were certainly correct compared with the other two diagnostic assays. **Conclusion:** It is highly recommended to incorporate PCR diagnostic assays alongside microscopic examination to evaluate the epidemiological status of equine piroplasmosis. Also, our study demonstrated that *T. equi* genotype A and genotype E are circulating among Egyptian horses.

Key words: Equine piroplasmosis, Microscopic examination, PCR, qPCR, *T. equi*

Introduction

Equine piroplasmosis, a tick-borne protozoal disease, poses a significant threat to the global equine industry. It is mainly caused by two apicomplexan parasites, *Theileria equi* and *Babesia caballi*, and is endemic in tropical, subtropical, and some temperate regions (Ozubek and Aktas, 2018; Souza *et al*., 2019). Other *Piroplasma* species, such as *Babesia ovis*, *Theileria annulata*, and *Theileria* sp. *africa*, have occasionally been reported in equine blood, suggesting possible cross-species transmission or atypical infections (Asif *et al*., 2020; Ceylan *et al*., 2021; Abdullah *et al*., 2022). Ticks from the genera *Dermacentor*, *Hyalomma*, and *Rhipicephalus* are the principal vectors of *T. equi* and *B. caballi* (Francisco *et al*., 2024; Sadeddine *et al*., 2025). The distribution of these vectors strongly influences the disease's prevalence and control (Bhagwan *et al*., 2015; Raza *et al*., 2024). In Egypt, species like *Hyalomma marginatum*, *H. anatolicum*, and *Rhipicephalus annulatus* have been implicated in *T. equi* transmission, with recent studies detecting parasite DNA in these ticks (Ramadan *et al*., 2024).

Economically, *T. equi* inflicts substantial losses on the equine sector by impairing animal movement and productivity due to the severe health effects it causes.

Equine theileriosis manifests through a range of nonspecific symptoms including anemia, icterus (jaundice), edema, loss of condition, and can lead to death (Salib *et al*., 2013). Animals that survive infection often carry the parasite with low-level parasitemia for life, making them a continuous source of infection to other animals in their vicinity (Ana *et al*., 2018). This persistent carrier state poses ongoing challenges for disease control and contributes to the economic burden on the equine industry (Malekifard *et al*., 2014; Mahmoud *et al*., 2016; Ahedor *et al*., 2023a; Altay *et al*., 2024).

In equine piroplasmosis, the level of parasitemia typically ranges between 1-5%, and in severe cases, may exceed 20% (Rothschild, 2013). Due to these low levels, especially in asymptomatic carriers, microscopy often lack sensitivity. In contrast, molecular diagnostics, particularly polymerase chain reaction (PCR) and real-time PCR, offer higher specificity and sensitivity for detecting parasitic DNA and capable of identifying parasitemia as low as 0.000001% (Alhassan *et al*., 2005; Kim *et al*., 2008; Davitkov *et al*., 2016). Genotyping based on 18S rRNA (SSU rRNA) has identified five *T. equi* genotypes (A-E) (Bhoora *et al*., 2009; Ahedor *et al*., 2023b). Previous studies have reported the molecular detection of *T. equi* in various areas across Egypt (Farah *et al*., 2003; Mahdy *et al*., 2016; Mahmoud *et al*., 2016; Elsawy *et al*., 2021; Abdel-Shafy *et al*., 2022; Ramadan *et al*., 2024). Research on this topic and its genotyping in Giza is limited. Additionally, previous reports on the occurrence of *T. equi* in this region (Mahmoud *et al*., 2020) reveal the need for more accurate diagnostic investigations of the parasite population.

This study aimed to detect *T. equi* in equines (n=116) from Giza Governorate using highly sensitive real-time PCR (qPCR). The qPCR results were compared with microscopy and conventional PCR to assess diagnostic performance. Additionally, genotyping was performed based on sequence analysis, contributing to the understanding of genetic diversity of *T. equi* in the region.

Materials and Methods

Ethical standards

This study was approved by the Ethical Research Committee of the Agricultural Research Center, Animal Health Research Institute, Egypt (ARC AHRI 1025). All procedures in this study accorded with Egyptian national animal welfare regulations and the research regulations.

Animals and sample collection

In the first half of 2019, we collected 116 blood samples from apparently healthy local breed horses raised in Nazlet El-Semman (29°58'46.5"N and 31°8'17EN), Giza Governorate, Egypt. The samples were drawn via the jugular venipuncture method into EDTA-coated tubes. Following collection, all samples were promptly transported to the Animal Health Research Institute (AHRI) in Dokki, Egypt for analysis.

Microscopic examination

Thin blood films were prepared from the whole blood samples and stained using Giemsa. These films were then examined under a light microscope at ×1000 magnification with an oil immersion lens to detect intraerythrocytic merozoites of *T. equi*.

DNA extraction

Genomic DNA was extracted from both microscopically positive and negative samples using the Thermo Scientific™ GeneJET Genomic DNA Purification kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA), following the manufacturer's guidelines. The extracted DNA was then stored at -80°C until further analysis.

Conventional PCR

The PCR assay was conducted using specific primers for *Piroplasma* spp., Bec-UF2 (Forward, 5´-TCG AAG ACG ATC AGA TAC CGT CG-3´) and Equi-R (Reverse, 5´-TGC CTT AAA CTT CCT TGC GAT-3´), targeting 392 bp fragment of *18S rRNA* gene of *Piroplasma* spp. (Alhassan *et al*., 2005). The reactions were performed using the GoTaq® G2 Flexi PCR kit (Promega, Madison, USA) in a total volume of 25 µL. This included 10 µL of 5X Green GoTaq® Flexi Buffer, 2 µL of 25 mM $MgCl_2$ solution, 1 µL of PCR Nucleotide Mix (dNTPs) at 10 mM each, 1 µL of a 10 pmol each primer, 5 µL of template DNA, and 6 µL of DNase/RNase free water. Thermal cycling conditions were set at 95°C for 5 min, followed by 35 cycles of 96°C for 1 min (denaturation), 60°C for 1 min (annealing), and 72°C for 1 min (extension), with a final extension at 72°C for 5 min, and a holding stage at 4°C. The PCR products were analyzed on a 1.5% ethidium bromide-stained agarose gel, running at 80 V for 40 min, and visualized using a Gel Documentation System (XR, Bio-Rad, Vizrt, UK). Positive samples for *Piroplasma* spp. were identified by the presence of 392 bp bands. PCR was meticulously performed, integrating robust protocols for contamination surveillance, which included the inclusion of both negative and positive controls. The positive control used in PCR comprised a sample known to be positive for the target pathogen, obtained from our laboratory. Furthermore, prior to its use, it underwent confirmation through sequencing.

Real-Time PCR (qPCR)

The qPCR assay was conducted following the protocol outlined by Kim *et al*. (2008), using specific primers and a probe targeting the *18S rRNA* gene of *T. equi*. The forward primer (Be18SF) had the sequence 5´-GCG GTG TTT CGG TGA TTC ATA-3´, the reverse primer (Be18SR) had the sequence 5´-TGA TAG GTC AGA AAC TTG AAT GAT ACA TC-3´, and the TaqMan probe (Be18SP) had the sequence 5´-AAA TTA GCG AAT CGC ATG GCT T-3´. The probe was labeled

with a FAM (6-carboxyfluorescein) reporter dye at the 5´ end and a TAMRA (6-carboxy-tetramethylrhodamine) quencher dye at the 3´ end. The qPCR reaction mixture, totaling 20 µL, included 4 µL of Mix stable qPCR 5x (Promega, Madison, USA), 1 µL of each primer and 0.5 µL of the probe, 5 µL of DNA template, and 8.5 µL of nuclease-free water. The qPCR was performed in a 96-well optical plate using the Stratagene Mx3005P system (Agilent technology, Santa Clara, USA). The thermal profile for qPCR consisted of an initial activation step at 95°C for 15 min, followed by 45 cycles of denaturation at 95°C for 20 s, hybridization and extension and data collection at 55°C for 1 min. We included both negative control and positive control in the experiment, using the same positive sample as in the conventional PCR.

Sequencing and phylogenetic analysis

Three positive candidates were randomly selected as a representative for the collection sites, for the subsequent direct sequence. Additionally, following the purification of DNA from the agarose gel using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Leicestershire, Duren, Germany) following the provided protocol, the concentration and purity of the extracted DNA were assessed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Following selection, the amplicons were submitted to sequencing unit at Animal Health Research Institute.

The obtained sequences from the bi-directional sequencing were initially analyzed using SnapGene® Viewer software (GSL Biotech, LLC, Boston, USA) (https://www.snapgene.com/). The forward and reverse sequences were aligned and merged using MEGA 11 software to generate complete sequences for further analysis. The obtained sequences were compared with sequences deposited in GenBank using the BLASTn tool available at www.blast.ncbi.nlm.nih.gov/Blast. The alignment of the sequences to reference sequences reported in GenBank was performed using the ClustalW algorithm in MEGA 11 software. After the alignment, the sequences were trimmed, and a model test was conducted in MEGA 11 to determine the most suitable evolutionary model for the data. Based on the selected model, phylogenetic trees were constructed using the Maximum Likelihood method. The robustness of the tree topology was assessed using bootstrap values based on 1000 replicates, as described by (Kumar *et al*., 2018).

Statistical analysis

The obtained results were subjected to One-way analysis of variance (ANOVA) for comparison, with differences assessed using Fischer's least square difference test. Additionally, a Chi-square ($\chi 2$) test was employed to compare the results obtained from microscopy, PCR, and qPCR. Significance was determined by a P-value <0.05. Assessment parameters, including specificity, sensitivity, likelihood ratio, positive predictive value (PPV), and negative predictive value (NPV), were calculated following the guidelines outlined by Thrusfield and Christley (2018).

Results

The examination of Giemsa-stained blood smears detected *Theileria* infection in 19 equines (16.4%), while conventional PCR identified the infection in 34 equines (29.3%), and qPCR confirmed it in 50 equines (43.1%). Consequently, qPCR results exhibited a significantly higher prevalence compared to conventional PCR and microscopic examination, as depicted in Table 1. In microscopic examination, *T. equi* manifested as various shapes including small single round, double round, and single pyriform (Figs. 1A-B). Conventional PCR yielded PCR products of *T. equi* with a size of 392 bp (Fig. 2), whereas in qPCR, positive samples were identified by the presence of positive FAM signals, while negative samples displayed negative FAM signals. Positive results were indicated by an S-shaped amplification curve, with the intersection point between the amplification curve and the threshold line referred to as the cycle threshold (Ct). The Ct values of our positive samples ranged from 20 to 30 (Fig. 3). Sequencing and phylogenetic analysis of our PCR-positive samples identified them as belonging to Genotypes A (Accesssion No. PP937599) and E (Accesssion No. PP937597 and PP937598), with sequence similarities ranging between 97-99% compared to the *T. equi* sequences deposited in GenBank. These results confirm the prevalence of these genotypes among the local equine population, as detailed in Fig. 4, which visually represents the genetic relationships and alignment with known GenBank entries.

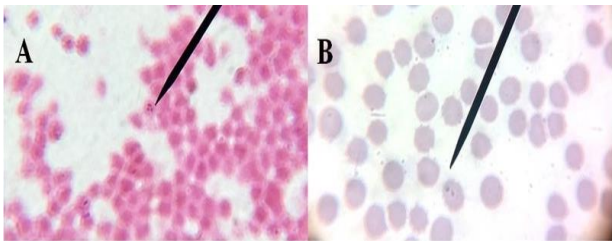

Fig. 1: Microscopically Giemsa-stained blood smear showed different forms of *T. equi* merozoite inside RBCs (×1000)

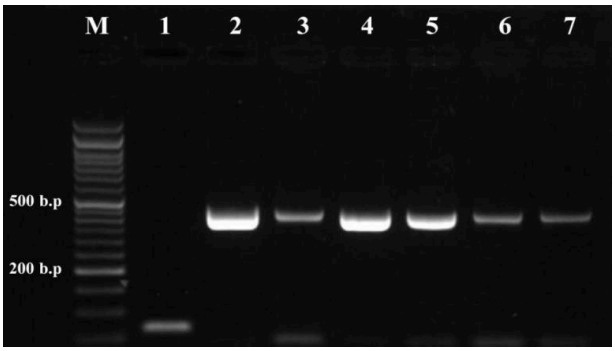

Fig. 2: Results of conventional PCR amplification for detection of *T. equi* at 392 bp fractionated on 1.5% agarose gel. Lane M: 50 bp DNA ladder. Lane 1: Negative control, Lane 2: Positive control, and Lanes 3-7: *T. equi* field positive samples

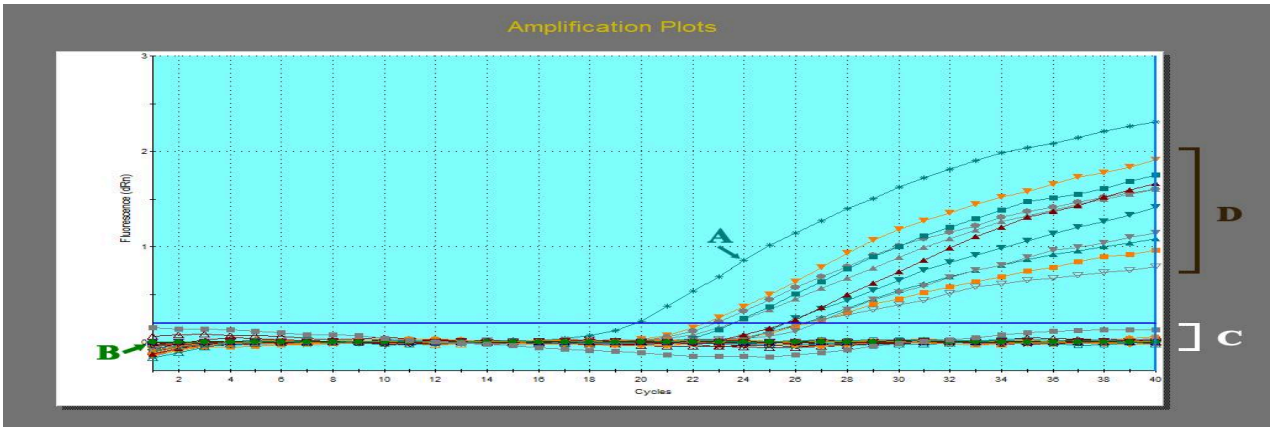

Fig. 3: qPCR results for *T. equi*. (**A**) Positive control, (**B**) Negative control, (**C**) Negative field samples, and (**D**) Positive field samples

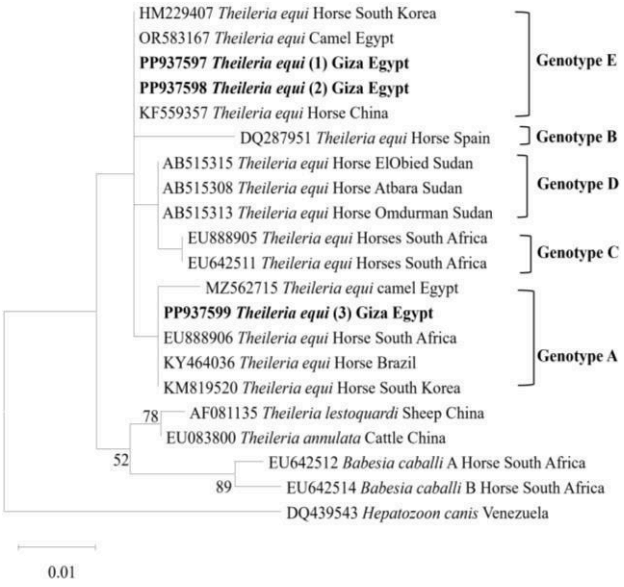

Fig. 4: The phylogenetic analysis for identification of *T. equi* based on *18S rRNA* gene sequences. The Maximum Likelihood phylogenetic tree construction and bootstrap analysis (1000 replicates) were performed according to the Jukes-Cantor distance model. The obtained sequences in this study are marked with bold face font (scale bar, 0.01 "Represents 0.01 nucleotide substitutions per site")

Table 1: Infection rate of *T. equi* among Egyptian equines by microscopic examination, conventional PCR and qPCR

Techniques	Number of infected samples	%
Microscope	19/116^{c}	16.4
Conventional PCR	34/116^{b}	29.3
Real-Time PCR (qPCR)	50/116^{a}	43.1

Data are presented as samples. Total infected samples/total number of samples and total percent of infected. Present superscript letters ($^{a, b, c}$) differ significantly

Table 3: Estimation of the likelihood ratio (LR) for comparison between microscopic examination, PCR, and qPCR for detection of *T. equi*

Techniques	Likelihood ratio +ve (LR+)	Likelihood ratio -ve (LR-)
Microscopic examination	Infinite (Inf)	0.62
Conventional PCR	Infinite (Inf)	0.32
Real-Time PCR (qPCR)	Infinite (Inf)	0.00

The evaluation parameters, including specificity, sensitivity, negative predictive value (NPV), and positive predictive value (PPV) are detailed in Table 2. This table indicates that all three diagnostic methods - microscopic examination, conventional PCR, and qPCR - achieved a specificity of 100%. However, qPCR demonstrated superior sensitivity at 100%, significantly higher than conventional PCR at 68% and microscopic examination at 38%. The results for the Likelihood Ratio are presented in Table 3.

Discussion

The present study emphasizes the remarkable efficacy of the qPCR assay in detecting *T. equi*, achieving a sensitivity of 100%, compared with 68% for conventional PCR and 38% for microscopic examination.

In this study, the prevalence of *T. equi* as detected by microscopic examination was 16.4% (19 out of 116), aligning closely with previous findings within Egypt - 19.8% by Radwan (2009), 13.9% by Ibrahim *et al.* (2011), 18% by Mahmoud *et al.* (2016), and 20.3% by Elsawy *et al.* (2021). However, these figures were lower than the 34% reported by 38.9% by Farah *et al.* (2003) and Salib *et al.* (2013). Internationally, the prevalence reported in this study was higher compared with results

Table 2: Assessment of Giemsa-stained blood smears, conventional PCR and qPCR for detection of *T. equi* amongst Egyptian equines

Techniques	Total	TP	TN	FP	FN	Sensitivity	Specificity	PPV	NPV
Microscopic examination	116	19	66	0	31	38	100	100	68.04
Conventional PCR	116	34	66	0	16	68	100	100	80.49
Real-Time PCR (qPCR)	116	50	66	0	0	100	100	100	100

TP: True positive, TN: True negative, FP: False positive, FN: False negative, PPV: Positive predictive value, and NPV: Negative predictive value

from other countries, such as 3.8% in Iran by Abedi *et al*. (2015), 6.3% by Malekifard *et al*. (2014), and 4.2% in India by Sumbria *et al*. (2015). In this study, the detection of *T. equi* using conventional PCR targeting the *18S rRNA* gene revealed a prevalence of 29.3% (34/116). This prevalence was slightly higher than the 26% reported by Ibrahim *et al*. (2011) in Egypt and Davitkov *et al*. (2016) in the Central Balkans, both using the same gene. However, it was somewhat lower than the 36.4% prevalence reported by Mahmoud *et al*. (2016) in Egypt using the *ema-1* gene. Using the *18S rRNA* gene for detection, the real-time PCR (qPCR) results in this study revealed a prevalence of 43.1% (50/116). This finding is closely aligned with the 42% reported by Alanazi *et al*. (2014) in Saudi Arabia, higher than the 36.2% observed by Jaffer *et al*. (2009) but notably lower than the 80% reported by Bhoora *et al*. (2010) in South Africa, all of which also targeted the *18S rRNA* gene. The variance in prevalence rates across different studies could be attributed to factors like vector activity, environmental conditions, and whether the disease was in its acute or chronic stage at the time of sampling, as suggested by Mahdy *et al*. (2016), Elsawy *et al*. (2021) and Ahedor *et al*. (2023b). These discrepancies in detection rates highlight the variability in regional disease prevalence and the impact of differing diagnostic techniques.

The findings of this study highlighted the superior sensitivity of qPCR in detecting *T. equi* infections. These results are consistent with findings from previous studies such as those by Ibrahim *et al*. (2011), Malekifard *et al*. (2014), Mohamed *et al*. (2016) and Elseify *et al*. (2018), all of which have demonstrated the increased sensitivity of molecular diagnostic techniques over traditional methods for detecting *T. equi*. This underscores the importance of using advanced molecular techniques in epidemiological surveillance and diagnostic protocols to ensure accurate detection, particularly in asymptomatic carriers. Notably, the negative predictive value (NPV) of qPCR reached 100%, significantly surpassing the NPVs of microscopic examination (68.0%) and conventional PCR (80.5%). These results demonstrate qPCR's unparalleled ability to correctly identify negative cases, making it a particularly reliable diagnostic tool in the assessment of equine theileriosis. Given these findings, it is advisable to employ a combined approach of PCR techniques alongside microscopic examination to enhance diagnostic accuracy and better understand the epidemiology of equine theileriosis, particularly in identifying asymptomatic carriers.

In the present study, the likelihood ratios for positive samples (LR+) were found to be infinite for all three techniques, indicating their ability to effectively detect true positives without generating false positives. Conversely, for negative samples (LR-), the values were 0.62, 0.32, and 0.00 for microscopic examination, conventional PCR, and qPCR, respectively. The LR+ serves as a quantitative measure of the strength of a positive result in a diagnostic test. Ideally, a perfect diagnostic test would yield an LR+ of infinity, accurately identifying all true positives while producing no false positives. Similarly, the ideal diagnostic test would yield an LR- of zero, detecting all true negatives without any false negatives. Following this principle, qPCR emerges as the most reliable technique for diagnosing *T. equi*, followed by conventional PCR and microscopic examination, in descending order of efficacy (Thrusfield and Christley, 2018).

By sequencing and phylogenetic analysis of our PCR-positive samples identified them as belonging to genotypes A and E, with sequence similarities ranging between 97-99% compared with the *T. equi* sequences deposited in GenBank. A previous study in Egypt reported *T. equi* genotype A circulating among Egyptian equines (Elsawy *et al*., 2021) while *T. equi* genotype C was reported as *T. haneyi* that is supposed to be a subgroup of *T. equi* genotype C (Knowles *et al*., 2018; Bhoora *et al*., 2020). *Theileria equi* genotype E, to our knowledge, is recorded for the first time among the equine population in this study after its detection in camels (Amer *et al*., 2024). In India the *T. equi* type A was mostly detected (Kumar *et al*., 2020; Maharana *et al*., 2024). Otgonsuren *et al*. (2024) recorded *T. equi* type A in Mongolian horses while genotype E was recorded as the dominant genotype in China (Zhang *et al*., 2023). These results confirm the prevalence of these genotypes among the local equine population. However, the confirmation of these identities would require amplification and sequencing of the entire *18S rRNA* gene. Our study has a notable limitation: while we focused on detecting the *T. equi* in blood samples, we did not collect anamnestic data for each individual animal. These limitations restrict our ability to evaluate potential risk factors and host-pathogen interactions comprehensively. We acknowledge this limitation and plan to address it in future research to ensure a more thorough and insightful analysis.

In conclusion, our study highlights the importance of using sensitive molecular diagnostic techniques, particularly qPCR, for the accurate detection of *T. equi* infections in equine populations. The superior sensitivity and negative predictive value of qPCR compared with conventional methods underscore its efficacy in both identifying true positives and ruling out false negatives. Furthermore, genotyping analysis revealed the presence of genotype A and genotype E strains, providing valuable insights into the genetic diversity of *T. equi* and first-time reporting of genotype A among the studied population. By incorporating these advanced techniques into routine surveillance and diagnostic protocols, we can better understand its epidemiology, especially in detecting asymptomatic carriers, managing and control this significant tick-borne disease, ultimately safeguarding the health and well-being of equines worldwide.

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Conflict of interest

The authors declare that they have no competing interests.

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