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RESEARCH ARTICLE

Epidemiology and Diagnostic Performance of *Theileria equi* Infection in Equids: a Multi-Method Study with Climatic and Management Risk Factors in Selected Districts of Central Punjab, Pakistan

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ABSTRACT

This study provides insights into multi-method epidemiological dynamics and performance of diagnostic tests in horses with associated risk factors of theileriosis. We analyzed 1,414 blood samples collected monthly over one year across multiple regions, assessing seasonal trends, geographical variations, environmental drivers, and diagnostic performance. Overall, microscopy detected *Theileria equi* in 8.42% of samples, ELISA seroprevalence was 19.3%, and PCR confirmed infection in 14.4%. qPCR peaks were observed in March (30.0%; 95% CI: 22.6–38.6%) and October (26.4%; 95% CI: 19.1–35.3%), whereas the lowest prevalence was recorded in April (4.7%; 95% CI: 2.4–9.0%). Seasonally, the highest prevalence occurred in fall (20.1%) and spring (12.8%), both significantly higher than winter (7.8%; P<0.001). Geographically, MBD/Head Faqerian (28.3%; 95% CI: 21.9–34.7%) and Sargodha (27.2%; 95% CI: 20.9–33.5%) were identified as major hotspots of prevalence. Beta regression revealed temperature as key predictor with 0.69% increase per 1°C increase (P<0.001), While high humidity (>60%) and heat waves increased the risk by 0.22% and 1.98%, respectively. The Bayesian latent class model estimated the sensitivity and specificity of PCR at 86.2% and 94.5%, respectively, while ELISA showed a sensitivity of 78.5% and specificity of 88.2%. Microscopy demonstrated comparatively lower sensitivity (65.4%) but higher specificity (96.1%). In conclusion, theileriosis revealed strong association with climate-density interactions, spring/fall peaks and rapidly increasing transmission. The study recommends adopting pre-peak acaricide treatments in March and October, managing hotspot density, and using dual ELISA/PCR testing to enhance accuracy in mitigating this issue.

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INTRODUCTION

Since their domestication, horses (*Equus caballus*) have been integral to human civilization, serving roles in trade, warfare, agriculture, and recreation. In developing nations, horses remain essential for agriculture and transportation, while in developed countries, they are predominantly used for sports and recreational activities. There are an estimated 116 million equines (donkeys, horses, and mules) globally, with 36 million in the 38

lowest-income countries (FAO, 2025). In Pakistan, the estimated horse population is about 0.4 million (Government of Pakistan, 2024). These horses are primarily utilized for racing, cart pulling, and riding (Gould *et al.*, 2025), with occasional use in agriculture and tent pegging competitions. Pakistan's position in the subtropical zone along the lush Indus River plains in South Asia has blessed the country with a variety of vibrant agro-ecological regions, each with its own unique climate and water resources. This agricultural landscape provides a favorable environment for ticks to thrive, potentially transmitting diseases to both livestock and humans (Hussain *et al.*, 2023).

Theileria (*T.*) *equi* is an obligate hemoprotozoan parasite transmitted by ticks and classified in the phylum Apicomplexa (Amiri *et al*., 2020; Bělková *et al.*, 2021; Kalantari *et al.*, 2022; Ahedor *et al.*, 2023a; Altay *et al.*, 2024). It is a major causative agent of equine piroplasmosis in tropical and subtropical regions. Theileriosis, an important tick-borne disease affecting equine populations worldwide, has considerable economic impacts (Bělková *et al.*, 2021; Altay *et al.*, 2024; Hermans *et al.*, 2025). The life cycle of *T. equi* involves two developmental stages within the host's body. During the initial phase of the illness, it infects white blood cells prior to red blood cells, leading to a persistent infection which is believed to be more aggressive than that caused by *Babesia caballi* (Ramadan *et al.*, 2024).

The classification of *T. equi* has been the subject of debate since its detection. Earlier, it was labeled *B. equi*; later, it was reclassified because it infects white blood cells rather than red blood cells (Mehlhorn and Schein, 1998). Recent phylogenetic analyses have identified four distinct clades of *T. equi*, designated as A, B, C, and D genotypes based on the hypervariable region of the 18S rRNA gene (Kappmeyer *et al.*, 2012; Ahedor *et al.*, 2023b).

In Asia, *T. equi* infection has been reported to be endemic in Pakistan, Central Mongolia, China, and India (Hussain *et al*., 2014; Maharana *et al.*, 2024; Otgonsuren *et al.*, 2024; Qin *et al.*, 2025). The Haemaphysaloides have been demonstrated as a potential vector for transmission of *T. equi* in Mongolia and China, but no such molecular evidence on ticks has been reported from other Asian countries. In Pakistan, *T. equi* infection is prevalent among working horses, adversely affecting their health and productivity (Afridi *et al.*, 2017; Aziz *et al.*, 2025). Species-specific PCR techniques primarily targeting the EMA-1 gene (Equine Merozoite Antigen-1) (Bhagwan *et al.*, 2015; Mège *et al.*, 2026) have been employed for the diagnosis of *T. equi*-infected equids in India. While PCR-based detection of *T. equi* has been reported in a limited number of studies in Pakistan, e.g., Ali *et al*. (2021), it has not yet been established as a routine diagnostic tool in national surveillance or veterinary laboratories. Moreover, only a limited number of microscopic and serological studies of equine theileriosis in Pakistan have been conducted (Afridi *et al.,* 2017). Additionally, past studies investigating the risk factors associated with equine theileriosis were limited to specific regions and had smaller reference populations of horses than the current study (Hussain *et al*., 2014; Afridi *et al*., 2017). Therefore, the present study was designed to investigate the epidemiology of *T. equi* infection in equids from selected districts of Central Punjab, Pakistan, using multiple diagnostic methods.

MATERIALS AND METHODS

Sampling strategy: A monthly repeated cross-sectional prevalence survey was designed to detect positive cases of theileriosis and identify risk factors associated with the disease. The study period was one year (January 2024 to December 2024). A total of 1,414 blood samples were collected from equids in the districts/tehsils of Sargodha, Bhalwal, Kot Momin, Shahpur, Sillanwali, Bhera, Sahiwal, and Mandi Bahauddin in Central Punjab, Pakistan (Table 1), assuming a 20% prevalence of an unknown disease in the equine population, with a 95% confidence interval (CI). The primary criterion for the sampled horses was the presence of ticks. All safety and ethical protocols were followed to prevent any accidental injury during animal handling and blood collection. Blood samples were obtained from the jugular vein and collected in two vacutainers: one for DNA extraction and hematological analysis, and the other for serum/ biochemical analysis. The samples were stored in ice boxes and immediately dispatched to the laboratory for examination. The samples were collected to assess the animal's disease status. Every sample also included a questionnaire with information on risk factors for theileriosis in horses, including month, age, sex, breed, area, density of equine holdings, body condition, nutrition, concentrate feeding, management, and tick infestation. Details of the study area, including its coordinates, elevation, and sample size, are provided in Table 1.

Table 1: Geographical coordinates and sampling summary of study locations

Area	Latitude	Longitude	Elevation from Sea	Samples Collected
Sargodha	32.082	72.6691	190	180
Kot Momin	32.1892	73.0286	205	173
Bhalwal	32.2723	72.9043	187	177
Shahpur	32.2676	72.4685	193	178
Sillanwali	31.8260	72.5396	184	177
Sahiwal	31.9735	72.3301	189	177
Bhera	32.4833	72.9167	192	172
Mandi Bahaudin / Head Faqerian	32.1614	73.0500	200	180

Blood smear examination: Thin and thick blood smears were prepared, stained with Field and Giemsa stains, air-dried, and then fixed in methanol. Blood smears were examined for protozoa under the oil-immersion (100x) lens by three experienced observers following the protocol of Zajac *et al*. (2021). The examination fields were chosen at the periphery of stained blood smears and at least 6 fields were examined for the presence of *T. equi* or other parasites. Case definitions and hemoprotozoan positivity rates for each diagnostic test are provided in Table 2.

Enzyme-Linked Immunosorbent Assay (ELISA): All positive, doubtful, and random negative samples in protozoology blood smear examination were subjected to a commercially available ELISA kit (BioStone Animal Health-AsurDx™ *T. equi* Antibody Test Kit, USA). Kit was used according to the manufacturer's instructions.

These assays detected *T. equi* IgG antibodies in horse serum/plasma using an enzyme-based immunoassay screening method. The optical density values were measured through an ELISA plate reader at 450nm, and the results were obtained using the following formula:

$$\text{Percent Positivity (PP)} = \frac{\text{OD450 Test Sample}}{\text{Mean OD450 Positive Control}} \times 100$$

All samples producing ≥60% PP were regarded as positive, whereas those with values <60% were considered negative.

Table 2: Case definitions and hemoprotozoan positivity rates for each diagnostic test

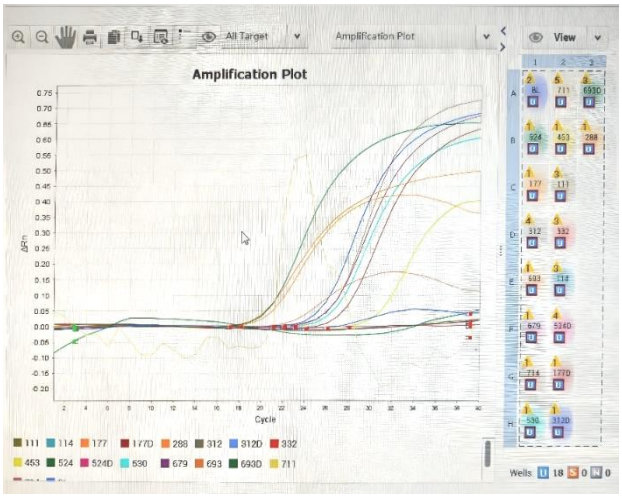
Test	Target	Interpretation	Positivity (%; 95% CI)
Microscopy (any piroplasm)	Parasite in RBCs	Active parasitemia	20.23 (18.2–22.4)
Microscopy (*T. equi* specific)	*T. equi* merozoites	Active *T. equi* infection	8.42 (7.0–9.9)
ELISA (*T. equi*)	Anti-*T. equi* antibodies	Past or present exposure	19.3 (17.3–21.4)
PCR (*T. equi*)	*T. equi* DNA	Active *T. equi* infection	14.4 (12.6–16.3)

Molecular detection: A DNA extraction kit (GeneAll, Exgene™, Catalog # 105-101) was used for the extraction of DNA from all blood samples according to the manufacturer's instructions, and the samples were subjected to SYBR green Quantitative Polymerase Chain Reaction (qPCR) assay targeting the 18S rRNA gene using 5'-CATCGTTGCGGCTTGGTTGG-3' and 5'-CCAAGTCTCACACCCTATTT-3' primers (Sumbria *et al.*, 2016)). PCR cycling conditions were 50°C for 2min, followed by 95°C for 10min and then 40 cycles of 95°C for 15sec and 60°C for 1min, as shown in Fig. 1.

Statistical Analysis: All analyses were performed using R version 4.2.0. and prevalence with 95% confidence intervals was calculated for each diagnostic test. We used the chi-square test followed by the pairwise Z-test with Holm-Bonferroni correction for data analysis. Differences in proportion were quantified using Cohen's h effect sizes with 95% CI reported for each estimate. A Bayesian latent class model (BLCM) was used with 95% credible intervals to detect sensitivity and specificity for all diagnostic tests, along with true prevalence. Mixed-effects logistic regression evaluated host risk factors (sex, age, co-housing), reporting odds ratios (95% CI) and likelihood-ratio P-values. For climate and density predictors (temperature, humidity, precipitation, heatwave (≥3 consecutive days ≥40°C), density), a beta regression was used. Assumptions were checked (VIF<5, residual plots). The temperature density interaction was tested separately. Coefficients are interpreted as absolute percentage-point changes after back-transformation. Model fit was evaluated using Pseudo-R^2 and likelihood ratio tests. Significance was set at α=0.05, adjusted for multiple comparisons.

RESULTS

Prevalence of *T. equi:* A total of 1,414 samples were analyzed in this study, and 20.23% of the sampled animals were positive for blood protozoan, including *Theileria* spp., *Babesia* spp., *Anaplasma* spp., and *Trypanosoma* spp. (286/ 1414) (Fig. 2). The overall prevalence of *T. equi*, mixed infection (*Babesia and Theileria* spp.), and other blood protozoa (anaplasmosis, trypanosomiasis, etc.) were 8.42%, 9.48%, and 2.3% (119, 134, and 33 samples, respectively) on microscopic examination (Fig. 2). However, more sensitive and specific diagnostic tests revealed higher rates, ELISA detected *T. equi* from 19.3% (273/1414) samples, and PCR confirmed active infection of 14.4% (203/1414) (Fig. 2). These results demonstrate that *T. equi* is endemic in the area, and combined diagnostic approaches (microscopy, serology, and molecular methods) are needed for accurate surveillance and control programs.



Well No	CT Values	Result
A3	40.00	Negative Control
B3	19.140	Positive Control
A1	40.00	Negative
B1	31.379	Negative
C1	30.346	Positive
D1	40.00	Negative
E1	23.797	Positive
F1	25.748	Positive
G1	26.268	Positive
H1	26.285	Positive
A2	40.00	Negative
B2	30.640	Positive
C2	40.00	Negative
D2	40.00	Negative
E2	40.00	Negative
F2	40.00	Negative
G2	27.244	Positive
H2	24.974	Positive

Fig. 1: Graphical representation of qPCR of equine samples.

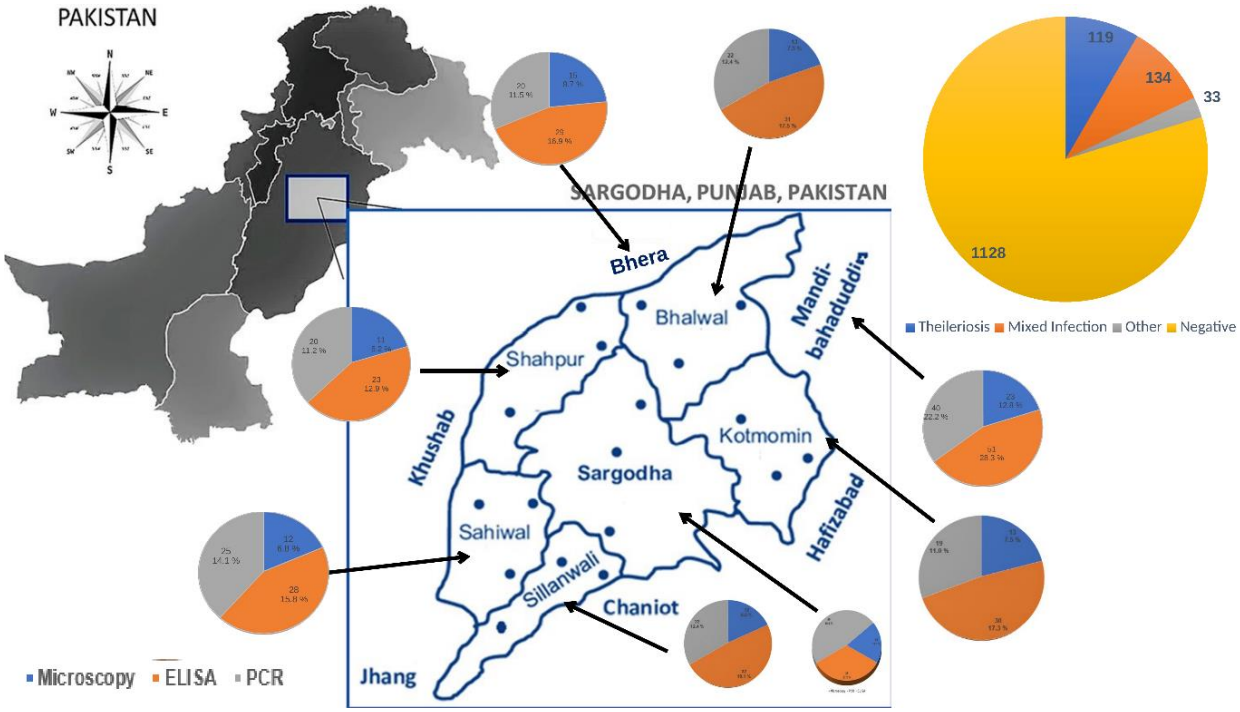


Fig. 2: Study areas and epidemiology of tick-borne diseases.

Host-level risk factors: Samples were examined for host-level associated risk factors, and a mixed effect logistic regression was performed to evaluate them; no statistically significant association was found for any examined factor (Table 3). Moreover, the odds ratio (OR) for males vs. females was 0.99 (95% CI: 0.72-1.36, P = 0.95). Older equids (>4 years) had an OR of 1.55 (95% CI: 0.92-2.62, P = 0.10) compared to younger equids (≤4 years). Co-housed animals vs. separate housing showed an OR of 0.73 (95% CI: 0.48-1.11, P=0.14). The intraclass correlation coefficient (ICC) for the region was 0.08, indicating that only 8% of the variance in infection status was attributed to regional differences. Overall, none of the evaluated factors (sex, age, or contact with other animals) were significantly associated with *T. equi* infection in the studied population (Table 3).

Table 3: Prevalence odds ratio and host-level associated risk factors (Mixed effects logistic regression)

Risk factors		No. examined	Positive	Prevalence (%)	Adjusted Odds Ratio (95% CI)	P-value
Sex	Female	770	118	8.35	1.00 (ref)	-
	Male	644	99	6.93	0.99 (0.72–1.36)	0.95
Age	> 4 years (older)	786	140	17.95	1.55 (0.92–2.62)	0.1
	≤ 4 years (young)	628	77	12.15	1.00 (ref)	-
Housing	Co-housed	434	55	12.60	0.73 (0.48–1.11)	0.14
	Separate housing	980	162	16.50	1.00 (ref)	-

Monthly prevalence: Table 4A demonstrated the monthly variation in *Theileria* prevalence, which varied significantly (χ^2=128.3, df=11, P<0.0001). The highest prevalence was observed in March, with detection rates of 25.0% (95% CI: 18.0-33.3%) by microscopy, 40.0% (31.8-48.8%) by ELISA, and 30.0% (22.6-38.6%) by qPCR. October showed comparable prevalence rates of 16.4% (10.6-24.5%), 30.0% (22.4-39.0%), and 26.4% (19.1-36.3%) by microscopy, ELISA, and qPCR, respectively and did not differ significantly from March (P=0.18). In contrast, the remaining months demonstrated comparatively moderate prevalence levels (Fig. 3). The lowest prevalence was recorded in April, with rates of 3.5% (1.6-7.5%) by microscopy, 11.2% (7.3-16.8%) by ELISA, and 4.7% (2.4-9.0%) by qPCR. Pairwise post-hoc comparisons with Holm-Bonferroni correction (α=0.0018) confirmed that March and October were significantly higher than all other months except (P=0.18 for March vs. October) (Table 4A).

Table 4A: Monthly Prevalence of *Theileria equi* in equines

Variable level	Microscopic parasitemia (%; 95% CI)	Prevalence (%; 95% CI)			Adjusted P- Value
		Microscopy	ELISA	PCR	
Mar	43.3 (34.5 - 52.6)	25 (18.2 -33.3)	40 (31.8 - 48.8)	30.0 22.6 - 38.6	<0.001*
Apr	6.5 (3.6 - 11.3)	3.5 (1.6 - 7.5)	11.2 (7.3 - 16.8)	4.7 2.4 - 9.0	<0.001*
Oct	37.3 (28.6 - 46.8)	16.4 (10.6- 2 4.5)	30 (22.4 - 39.)	26.4 19.1 - 35.3	0.18

*Holm-Bonferroni correction for 66 comparisons (α = 0.0018).

Diagnostic test performance: A Bayesian latent class model (BLCM) was used to estimate the sensitivity and specificity of the three diagnostic tests (microscopy, ELISA and PCR) simultaneously, as well as the true prevalence of infection in the study population. The diagnostic performance analysis revealed the differences in test characteristics for *Theileria* detection. Results showed that PCR had the highest sensitivity (86.2%) and specificity (94.5%), making it the best confirmatory test. ELISA showed moderate sensitivity (78.5%) and specificity (88.2%), making it suitable for screening of infection. Microscopy had very high specificity (96.1%) but low sensitivity (65.4%), meaning a positive result is reliable, but a negative result does not rule out infection

(Fig. 5). The true prevalence was estimated at 18.2%. All tests had strong negative predictive values (>92%), useful for excluding disease when negative (Fig. 5).

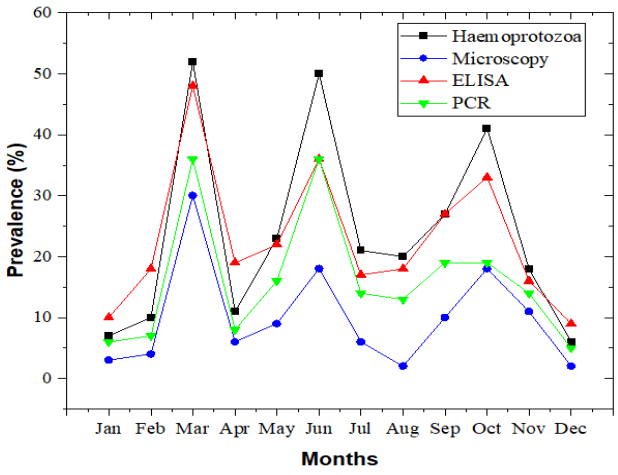


Fig. 3: Graphical representation of *T. equi* prevalence with different Diagnostic Tests and microscopic prevalence of hemoprotozoan.

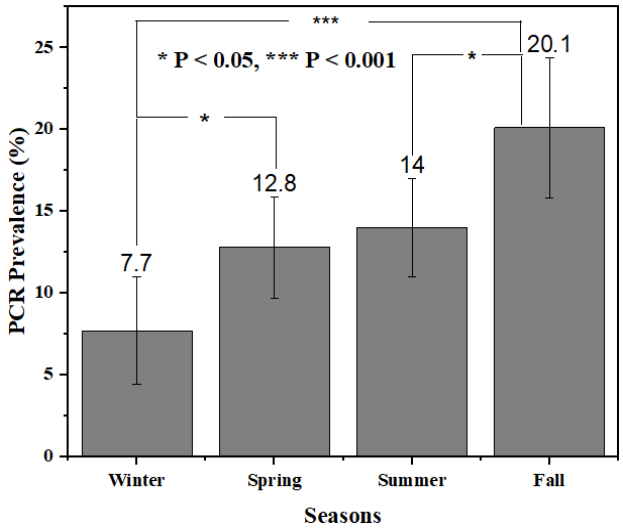


Fig. 4: Graphical representation for comparison of different diagnostic tests. Bars represent prevalence estimates and error bars indicate 95% confidence intervals. Significant pairwise comparisons after Bonferroni adjustment are indicated by asterisks (* P<0.05; *** P<0.001).

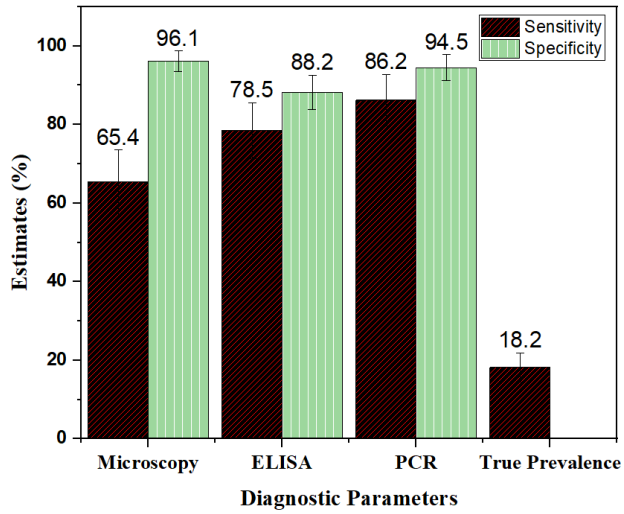


Fig. 5: Graphical representation of BLCM comparison for different diagnostic tests.

Seasonal prevalence of *T. equi* (PCR): The seasonal analysis of *Theileria* prevalence revealed significant variations in infection rates round the year. Fall showed the highest prevalence of 20.1% (95% CI:15.8-24.4%), followed by Spring 12.8% (9.7-15.9%), and Summer 14% (11-17%). Winter had a lower prevalence of 7.7% positivity (4.4-11%) and used as standard for comparison with other seasons. Overall chi-square value for seasonal differences yielded: χ^2=48.6, df=3, P<0.001. Pairwise post-hoc comparisons with Holm-Bonferroni correction showed that Fall prevalence was significantly higher than Winter Δ=12.4%, (8.2-16.6%, adjusted P<0.001) and significantly lower in Summer Δ=-6.1% (-10.4 to -1.8%, adjusted P=0.018). Spring vs Winter prevalence was also significant Δ=5.1% (1.4-8.8%, adjusted P=0.029), however, Spring vs. Summer prevalence was found non-significant Δ=-1.2% (-5.4 to 3.0%, P=0.65) for PCR (Table 4B). Moreover, detailed results of pairwise post hoc comparisons are mentioned in Table 5.

These seasonal patterns were statistically highly significant (P<0.001) and demonstrated clear temporal trends in disease risk, with Fall and Summer representing critical periods as compared to Spring and Winter for surveillance and control measures. However, seasonal pairwise post hoc comparisons (Bonferroni-adjusted) revealed that *Theileria* prevalence was consistently higher in Fall and Spring compared to Winter across all diagnostic modalities (Fig. 4). The data support the need for seasonal adaptation of prevention strategies, with particular emphasis on the high-risk Fall period when infection rates are at their peak across all diagnostic methods.

Regional prevalence of *T. equi* (PCR): The regional analysis revealed significant variation in *Theileria* prevalence across study locations (χ^2=52.1, df=7, P<0.001). The highest infection rates were observed in Mandi Bahaudin/Head Faqerian (28.3%, 95% CI:23.3-36.7%) and Sargodha (27.2%, CI:20.9-33.5%). In contrast, Shahpur 12.9% (8.3-17.5%) and Sahiwal 15.8% (10.9-20.7%) showed the lowest PCR positivity rates. Intermediate prevalence was observed in Kot Momin, Bhalwal, Sillanwali and Bhera with overlapping confidence intervals suggesting similar transmission intensity in these areas. These trends were consistent across all diagnostic methods, such as MBD/Head Faqerian exhibiting 12.8% (8.5-17.1%) *T. equi* microscopic positivity, 22.2% (16.4-28.0%). ELISA positivity (Table 4C and Fig. 6).

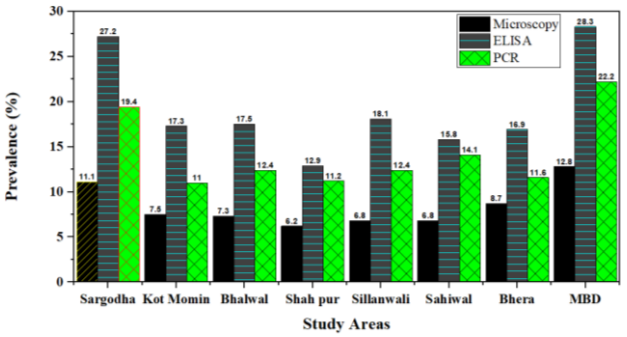


Fig. 6: Geographical prevalence representation of different diagnostic tests.

Table 4B: Seasonal Comparison of PCR positivity of *Theileria equi* in equines

Season	Seasonal PCR (%; 95% CI)	Comparison	Δ% (95% CI)	Adjusted P-value
Fall	20.1 (15.8–24.4)	Winter	+12.4 (8.2–16.6)	<0.001**
Spring	12.8 (9.7–15.9)	Winter	+5.1 (1.4–8.8)	0.029
Summer	14.0 (11.0–17.0)	Fall	–6.1 (–10.4 to –1.8)	0.018*
Winter	7.7 (4.4–11.0)		–	–
Spring		Summer	–1.2 (–5.4 to 3.0)	0.65

PCR (%) = PCR-positive prevalence of *Theileria equi*; CI = confidence interval; Δ% = absolute difference in prevalence between seasons. Adjusted P-values were obtained using Bonferroni-corrected pairwise comparisons. * P<0.05; ** P<0.001.

Table 4C: Regional Comparison of PCR positivity of *Theileria equi* in equines

Region	PCR+ (%; 95% CI)	vs MBD/HF	Adjusted P-value
MBD/Head Faqirian	28.3 (21.9–34.7)	Ref	–
Sargodha	27.2 (20.9–33.5)	–1.1 (–9.3 to 7.1)	1.0
Sillanwali	18.1 (12.8–23.4)	–10.2 (–17.8 to –2.6)	0.008
Bhalwal	17.5 (12.2–22.8)	–10.8 (–18.4 to –3.2)	0.006
Kot Momin	17.3 (12.0–22.6)	–11.0 (–18.6 to –3.4)	<0.001**
Bhera	16.9 (11.6–22.2)	–11.4 (–19.0 to –3.8)	<0.001**
Sahiwal	15.8 (10.9–20.7)	–12.5 (–20.2 to –4.8)	<0.001**
Shahpur	12.9 (8.3–17.5)	–15.4 (–22.8 to –8.0)	<0.001**

PCR+ = PCR-positive prevalence; CI = confidence interval; Ref = reference category (Mandi Bahauddin/Head Faqirian). Adjusted P-values were calculated using Bonferroni-adjusted pairwise comparisons. * P<0.05; ** P<0.001.

Table 5: Seasonal pairwise post hoc comparisons using Bonferroni-adjusted method

Comparison	Test	Δ% (95% CI)	P-value*	Cohen's h (95% CI)
Fall vs. Winter	Microscopy (all protozoa)	+18.1 (12.3–23.9)	<0.001**	0.82 (0.60–1.04)
	T. equi	+8.9 (5.1–12.7)	<0.001**	0.58 (0.37–0.79)
	ELISA	+9.0 (5.3–12.7)	<0.001**	0.55 (0.34–0.76)
	PCR	+12.4 (8.2–16.6)	<0.001**	0.68 (0.47–0.89)
Spring vs. Winter	Microscopy	+11.7 (6.4–17.0)	<0.001**	0.64 (0.43–0.85)
	T. equi	+6.2 (2.9–9.5)	0.003**	0.48 (0.27–0.69)
	ELISA	+5.6 (1.8–9.4)	0.012*	0.39 (0.18–0.60)
	PCR	+5.1 (1.4–8.8)	0.029*	0.36 (0.15–0.57)
Fall vs. Summer	Microscopy	+8.6 (2.9–14.3)	0.004**	0.41 (0.21–0.61)
	T. equi	+5.7 (2.0–9.4)	0.011*	0.44 (0.23–0.65)
	ELISA	+8.8 (3.5–14.1)	0.002**	0.50 (0.29–0.71)
	PCR	+6.1 (1.8–10.4)	0.018*	0.42 (0.21–0.63)
Spring vs. Summer	Microscopy	+2.2 (–3.1 to 7.5)	0.42	0.12 (-0.09 to 0.33)
	T. equi	+3.0 (–1.2 to 7.2)	0.12	0.22 (0.01–0.43)
	ELISA	+5.4 (1.2–9.6)	0.022*	0.32 (0.11–0.53)
	PCR	–1.2 (–5.4 to 3.0)	0.65	0.08 (-0.13 to 0.29)

Δ% = absolute difference in prevalence between seasons; CI = confidence interval; Cohen's *h* = standardized effect size for differences between proportions. * P<0.05; ** P<0.01 after Bonferroni adjustment.

Multivariable analysis for the prevalence of *T. equi*: The result of beta multivariate regression (logit link) is described in Table 6. The analysis revealed that average temperature, animal density, and humidity were the strong predictors of *Theileria* prevalence. Higher temperatures showed a particularly strong effect, each 1°C increase in average monthly temperature was associated with an absolute rise of 0.69 (0.41-0.97, P<0.001) percentage points in prevalence. This explains why May (33.5°C) and June (36.0°C) had such high infection rates, while cooler months like January (13.1°C) and December (13.6°C) showed significantly lower transmission. Heatwaves (≥40°C) further amplified risk, adding an absolute increase of 1.98% to prevalence (0.88-3.08 P=0.002), which aligns with the spikes seen in April and May. In unadjusted comparisons, high-density regions (MBD and Sargodha) had 12-15% higher prevalence than low-density areas. However, after adjusting for temperature, humidity, and heatwaves, the independent effect of high animal density was an absolute increase of 1.05 percentage points (0.62-1.48, P<0.001).

Humidity also played an important role, with higher humidity (>60%) increased the risk by 0.22% (0.09-0.35 P=0.003), particularly during July-September monsoons, when warm, moist conditions likely prolonged tick survival and growth, resulting in the highest prevalence of *T. equi* during these months.

Additionally, precipitation alone did not significantly affect prevalence (P=0.21), possibly because heavy rain both washes away ticks and creates humid microclimates, producing counteracting effects. The model's high explanatory power (R^2=0.73) confirms that temperature-driven transmission, animal density, and humidity are the dominant drivers of *Theileria* outbreaks (Table 6).

Table 6: Multivariable beta regression analysis of factors associated with PCR-positive proportions in the study regions

Predictor	Coefficient (β)^	95% CI	P-value	Significance
Avg Temp (°C)	0.69	0.41–0.97	<0.001**	Highly Significant
Humidity (%)	0.22	0.09 - 0.35	0.003*	Significant
Precipitation	-0.03	–0.08 to 0.02	0.21	Non-Significant
Heatwaves	1.98	0.88 - 3.08	0.002*	Significant
Animal density	1.05	0.62 - 1.48	<0.001**	Highly significant

^All coefficients are from beta regression with logit link, back-transformed to percentage point changes on the original 0–100 scale. * Significant at P<0.05; ** Highly significant at P<0.01.

DISCUSSION

Equine theileriosis is an emerging tick-borne disease that threatens horses' health, causing anemia, immunosuppression, and significant economic losses in the equine industry. Keeping into consideration the socio-economic importance of disease and its health impacts, the current study is designed to determine the prevalence of theileriosis in equines, assuming high prevalence of this infection in the study area. Though a number of studies have been published regarding the epidemiology of this disease in Pakistan, however, results are contrasting due to variations in the experimental design, sample population, and diagnostic testing (Hussain *et al.*, 2014; Nadal *et al.*, 2022; Hussain *et al.*, 2023; Raza *et al.*, 2024). Equine theileriosis is a globally prevalent disease, and infected animals are often asymptomatic carriers, spreading infection among the population. Due to low parasitemia in these animals, conventional methods (microscopic examination) often fail to precisely identify those carriers. However, tests that rely on identifying the genomic fingerprints or antibodies of pathogens have greater precision for diagnosing equine theileriosis (Soliman *et al*., 2021).

A similar study was conducted by Nadal *et al.* (2022) which highlighted the importance of data for monitoring cumulative exposure and identifying high-risk regions, which aligns with our approach. The study indicated lower sensitivity of complement fixation tests compared to modern ELISA and PCR. Therefore, this study investigated the prevalence of *T. equi* using multiple diagnostic tests and a larger sample size across all regions of the study area. The results of current study revealed an

overall 20.23% prevalence of protozoal infection, with 8.42% of *T. equi* on microscopic examination and 14.4%, 19.3% on PCR and cELISA, respectively, among the studied population. This divergence between tests advocates that microscopy alone could underestimate true prevalence and might failed to truly detect subclinical or chronic cases, however, due to its rapidity and cost effectiveness, microscopy is still being widely employed to detect blood protozoans (Kashif *et al.*, 2026). These findings also highlighted that *T. equi* is an important pathogen in the studied population, with nearly one out of every fifth studied animal had exposure of *T. equi* (ELISA) and about 14% had active infections (PCR). These results are similar to findings from previous studies (Bhagwan *et al.*, 2015; Ali *et al.*, 2021). However, our findings are significantly different when compared with the findings of other studies (Kumar *et al.*, 2004; Nehra *et al.*, 2024; Soliman *et al.*, 2025) which might be due to differences in diagnostic testing, sample population, and experimental design (preferably selection of tick-infested equids).

Statistical analysis of monthly prevalence revealed significant variations in infection rates throughout the year, with marked surges during specific months. March emerged as the period of highest parasitemia, with overall hemoprotozoan positivity of 43.3% (34.5-52.6%) and *T. equi* positivity of 25.0% (18.0-33.3%) by microscopy, 40.0% (31.8-48.8%) by ELISA, and 30.0% (22.6-38.6%) by PCR. A secondary, but significant, peak was observed in October, with 37.3% microscopic positivity (28.6-46.8%) and PCR detection in 26.4% (19.1-35.3%) horses. In contrast, April had the lowest infestation rate of 6.5% (3.6-11.3%), representing a 6-7-fold difference compared to the peak prevalence. These patterns of *Theileria* infestation stalwartly expressed a stratified diagnostic approach: staining for initial active cases, ELISA for surveillance/ screening, and PCR for confirmation of suspect cases. Additionally, our findings inform the timing of surveillance systems for preventive interventions before, during, or after peak infection risk periods, benefiting both clinical management and research on theileriosis.

However, the diagnostic test findings (Staining, ELISA, and PCR) indicate an effective testing strategy: starting with ELISA for initial screening due to its higher sensitivity, then confirming results with PCR because of its specificity. Microscopy remains a practical tool for screening or diagnosis but is less accurate and better suited as a field diagnostic method. These patterns of *Theileria* infestation expressed a stratified diagnostic approach which coincides with the findings of previous literature studies (Camino *et al.*, 2021; Idoko *et al.*, 2021).

The regional patterns were consistent irrespective of any diagnostic modality including microscopy, ELISA and PCR, and consistently identified MBD/ Head Faqerian and Sargodha as persistent hot spots. These findings revealed significant geographic heterogeneity for theileriosis. As noted by Nadal *et al*. (2022), such heterogeneity in piroplasm circulation can be linked to vector distribution (e.g., *Dermacentor*, *Rhipicephalus*, and *Hyalomma* species), which are influenced by land use, vegetation, and climate. In the same study (Nadal *et al.*, 2022) observed the temporal variations significantly increase *T. equi* seroprevalence over the years in France, which aligns with our findings that optimal temperature is associated with high prevalence. Furthermore, our results determine that Fall and Spring constantly had highest *Theileria* infestation and had significant statistical differences as compared to summer and winter in all tests. This consistency of patterns in all diagnostic approaches reinforces the validity of the observed seasonal trends. It is therefore concluded that temperature is a critical driver of infection and these findings are strongly supported by a trans-boundary study from Punjab, India, where the spatial distribution of *T. equi* showed a direct relationship with temperature and an inverse relationship with humidity (Sumbria *et al.*, 2016). Future studies in Pakistan should incorporate tick surveillance to confirm the role of specific vectors in driving the observed spatial patterns and to establish the association of management practices, breed, nutrition, and acaricide use within aggregate-level models.

Mixed effects logistic regression revealed non-significant host-level risk factor (sex, age, co-housed animals) trends contributing to *T. equi* prevalence. The data demonstrated considerable epidemiological trends, with a comparatively higher prevalence observed in older horses (17.95%) compared to younger animals (12.15%), in females (8.35%) relative to males (6.93%), and among co-housed horses than in isolated animals, although these differences were statistically non-significant ($P > 0.05$). The increased prevalence in older horses might be due to stress-associated recrudescence and lifelong carrier status, whereas the variation between sexes might be due to differences in animal utilization patterns and management practices. Furthermore, the relatively greater occurrence among co-housed animals might be associated with vector-mediated transmission and pathogen sharing, consistent with the findings of previous literature studies (Asgarali *et al.*, 2007; Pereira *et al.*, 2023; Kyari *et al.*, 2025).

Bayesian latent modeling showed that PCR was the most accurate test, while microscopy had excellent specificity but lower sensitivity; a positive microscopy result is reliable for diagnosis of active infection, but a negative result does not exclude the presence of disease. The estimated true prevalence of 18.2% lies between the raw microscopy with values of 20.23% and PCR with values of 14.4%, reflecting the imperfections of all tests, when performed individually. The importance of adopting a combined diagnostic approach is reinforced by the work of Nardini *et al*. (2022) in Italy, which concluded that ELISA should be used in conjunction with PCR and that the latter is crucial for confirming early infections.

Conclusions: This study was designed to detect positive cases of theileriosis and to identify predominant risk factors associated with the disease. The prevalence rate of *T. equi*, piroplasmosis, and other blood protozoa in the current study were 8.42%, 9.48%, and 2.3 % (119, 134, and 33 samples, respectively) on microscopic examinations. The prevalence of *T. equi* across the seasons (Fall, Spring, Summer, and Winter) was assessed using ELISA and qPCR. Overall, *T. equi* emerged as a key pathogen in the studied population, evidenced by seroprevalence approaching 20% and PCR-confirmed infection in 14% of animals. Consistent regional patterns

were observed across microscopy, cELISA, and PCR, indicating similar prevalence estimates among regions. Theileriosis showed marked seasonal variation, with significantly higher infestation during Fall and Spring as compared to Summer and Winter seasons. These findings demonstrated targeted tick control in high-density areas before heatwaves and humidity spikes which might effectively reduce infection rates. For precision planning, integrating tick surveillance data with current findings could further refine risk predictions. This proactive, evidence-based approach is essential to mitigate the clinical and hematobiochemical impacts of theileriosis and prevent the substantial economic losses it imposes on the equine industry.

Future recommendations: The study advocates implementing pre-emptive, seasonally targeted disease control strategies to reduce infection risk and improve equine health. Specifically, targeted acaricide application should be scheduled prior to the identified high-risk transmission periods of March and October to disrupt peak tick activity. Concurrently, in identified equine congregation hotspots, density management strategies such as rotational grazing and avoiding overcrowding must be enforced to mitigate the increased transmission linked to high host concentration. To enhance diagnostic accuracy and surveillance, a tiered laboratory approach is recommended. This involves using cELISA for sensitive serological screening, followed by confirmatory PCR testing, particularly for subclinical or ambiguous cases, while retaining microscopy as a vital, field-friendly tool in resource-limited settings. Furthermore, a dedicated national surveillance program for equine tick-borne diseases should be established, focusing on monitoring high-risk districts and seasonal windows to track epidemiological trends. To build a foundation for long-term resilience, future research should develop climate-based forecasting models to predict outbreak risks and pursue whole-genome sequencing of local *T. equi* strains to understand pathogen diversity. Additional studies should also aim to identify factors associated with disease susceptibility or resistance in local equine populations. By adopting these immediate, evidence-based interventions supported by sustained surveillance and strategic research, the significant clinical and economic impact of equine theileriosis can be substantially reduced. Therefore, future studies should integrate tick surveillance, host mobility patterns, and genomic characterization of circulating *Theileria* strains.

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Author's contribution: MSK, MI, SU, FR and RDM conceived and designed the research study. QB, MSK, MI, MT and FR executed the experiment and analyzed the samples. MSK, MI, QB and MT analyzed the data. Writing-Original draft by QB, RDM, BM, AHS and FF. All authors contributed to the manuscript and approved the final version for submission.

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REFERENCES

Afridi MJK, Mian AH, Saqib M, *et al*., 2017. Seroprevalence and risk factors for *Theileria equi* infection in equines from Khyber Pakhtunkhwa province, Pakistan. Iranian Journal of Parasitology 12:597–605.

Ahedor B, Sivakumar T, Valinotti MFR, *et al*., 2023a. PCR detection of *Theileria equi* and *Babesia caballi* in apparently healthy horses in Paraguay. Veterinary Parasitology: Regional Studies and Reports 39:100835. https://doi.org/10.1016/j.vprsr.2023.100835

Ahedor B, Otgonsuren D, Zhyldyz A, *et al*., 2023b. Development and evaluation of specific polymerase chain reaction assays for detecting *Theileria equi* genotypes. Parasites Vectors 16:435. https://doi.org/10.1186/s13071-023-06045-z

Ali S, Ijaz M, Farooqi SH, *et al*., 2021. Molecular characterisation of *Theileria equi* and risk factors associated with the occurrence of theileriosis in horses of Punjab (Pakistan). Equine Veterinary Education 33:75–83. https://doi.org/10.1111/eve.13161

Altay K, Erol U, Sahin OF, *et al*., 2024. Survey of tick-borne pathogens in grazing horses in Kyrgyzstan: phylogenetic analysis, genetic diversity, and prevalence of *Theileria equi*. Frontiers in Veterinary Science 11:1359974. https://doi.org/10.3389/fvets.2024.1359974

Amiri MS, Yaghfoori S and Razmi Gh, 2020. Molecular detection of *Theileria annulata* in dairy cattle and vector ticks in the Herat area, Afghanistan. Archives of Razi Institute 76(1):79–85. https://doi.org/10.22092/ari.2019.128212.1407

Asgarali Z, Coombs DK, Mohammed F, *et al*., 2007. Serological study of *Babesia caballi* and *Theileria equi* in Thoroughbreds in Trinidad. Veterinary Parasitology 144:167–171. https://doi.org/10.1016/j.vetpar.2006.09.015

Aziz KJ, Al-Barwary LTO, Issa NA, *et al*., 2025. Prevalence of *Theileria equi* and *Babesia caballi* and associated hemato-biochemical changes in racing horses in Erbil province, Iraq. Acta Parasitologica 70:38. https://doi.org/10.1007/s11686-024-00987-9

Bělková T, Bártová E, Řičařová D, *et al*., 2021. *Theileria equi* and *Babesia caballi* in horses in the Czech Republic. Acta Tropica 221:105993. https://doi.org/10.1016/j.actatropica.2021.105993

Bhagwan J, Kumar A, Kumar R, *et al*., 2015. Molecular evidence of *Theileria equi* infection in *Hyalomma anatolicum* ticks infested on sero-positive Indian horses. Acta Parasitologica 60. 322-329. https://doi.org/10.1515/ap-2015-0045

Camino E, Buendia A, Dorrego A, *et al*., 2021. Sero-molecular survey and risk factors of equine piroplasmosis in horses in Spain. Equine Veterinary Journal 53:771–779. https://doi.org/10.1111/evj.13348

FAO, 2025. FAO Statistical Database Website, Food and Agriculture Organization, Rome, Italy.

Gould S, Roy IR, Gabriel FD, *et al*., 2025. Equestrian sports safety: An update on safety equipment and event medical coverage. Current Sports Medicine Reports 24:11–17. https://doi.org/10.1249/jsr.0000000000001222

Government of Pakistan. 2024. *Pakistan Economic Survey 2023–24*. Finance Division, Economic Adviser's Wing, Islamabad, Pakistan.

Hermans L-M, Bonsergent C, Josson A, *et al*., 2025. Evaluation of *Theileria equi* vertical transmission rate and routes in a cohort of asymptomatic mares and their foals. Ticks and Tick-Borne Diseases 16:102432. https://doi.org/10.1016/j.ttbdis.2024.102432

Hussain MH, Saqib M, Raza F, *et al*., 2014. Seroprevalence of *Babesia caballi* and *Theileria equi* in five draught equine populated metropolises of Punjab, Pakistan. Veterinary Parasitology 202:248–256. https://doi.org/10.1016/j.vetpar.2014.01.026

Hussain N, Shabbir RMK, Ahmed H, *et al*., 2023. Prevalence of different tick species on livestock and associated equines and canine from different agro-ecological zones of Pakistan. Frontiers in Veterinary Science 9:1089999. https://doi.org/10.3389/fvets.2022.1089999

Idoko IS, Edeh RE, Adamu AM, *et al*., 2021. Molecular and serological detection of piroplasms in horses from Nigeria. Pathogens 10:508. https://doi.org/10.3390/pathogens10050508

Kalantari M, Sharifiyazdi H, Ghaemi M, *et al*., 2022. *Theileria equi* in the horses of Iran: Molecular detection, genetic diversity, and hematological findings. Veterinary Parasitology: Regional Studies and Reports 36:100792. https://doi.org/10.1016/j.vprsr.2022.100792

Kappmeyer LS, Thiagarajan M, Herndon DR, *et al*., 2012. Comparative genomic analysis and phylogenetic position of *Theileria equi*. BMC Genomics 13:603. https://doi.org/10.1186/1471-2164-13-603

Kashif M, Nasir A, Khan H, *et al*., 2026. Equine Piroplasmosis in Punjab, Pakistan: A clinico-epidemiological and molecular study with therapeutic insights. Egyptian Journal of Veterinary Sciences 0:1–12. https://doi.org/10.21608/ejvs.2026.442359.3277

Kumar S, Yokoyama N, Kim JY, *et al*., 2004. Expression of Babesia equi EMA-1 and EMA-2 during merozoite developmental stages in erythrocyte and their interaction with erythrocytic membrane skeleton. Molecular and Biochemical Parasitology 133:221–227. https://doi.org/10.1016/j.molbiopara.2003.10.010

Kyari F, Kayeri BK, Zango MK, *et al*., 2025. Seroepidemiology and risk factors associated with *Theileria equi* and *Babesia caballi* infections in horses from Borno and Yobe states, Nigeria. International Journal of Equine Science 4:21–29. https://doi.org/10.64292/ijes.159

Maharana BR, Ganguly A, Potliya S, *et al*., 2024. Molecular detection and characterization of prevailing *Theileria equi* genotype in equine from northern India. Research in Veterinary Science 173:105277. https://doi.org/10.1016/j.rvsc.2024.105277

Mège M, Berquier F, Bonsergent C, *et al*., 2026. *Theileria equi* sensu lato genetic diversity and phylogeny: new insights from mitochondrion, apicoplast and nucleus multilocus gene analysis. Ticks and Tick-Borne Diseases 17:102656. https://doi.org/10.1016/j.ttbdis.2026.102656

Mehlhorn H and Schein E, 1998. Redescription of *Babesia equi* Laveran, 1901 as *Theileria equi* Mehlhorn, Schein 1998. Parasitology Research, 84:467–475. https://doi.org/10.1007/s004360050431

Nadal C, Marsot M, Le Metayer G, *et al*., 2022. Spatial and temporal circulation of *Babesia caballi* and *Theileria equi* in France based on seven years of serological data. Pathogens 11:227. https://doi.org/10.3390/pathogens11020227

Nardini R, Cersini A, Pino LEBD, *et al*., 2022. Comparison of direct and indirect methods to maximise the detection of *Babesia caballi* and *Theileria equi* infections in central southern Italy. Ticks and Tick-borne Diseases 13:101939. https://doi.org/10.1016/j.ttbdis.2022.101939

Nehra AK, Kumari A, Moudgil AD, *et al*., 2024. Revisiting the genotypes of *Theileria equi* based on the V4 hypervariable region of the 18S rRNA gene. Frontiers in Veterinary Science 11:1303090. https://doi.org/10.3389/fvets.2024.1303090

Otgonsuren D, Amgalanbaatar T, Narantsatsral S, *et al*., 2024. Epidemiology and genetic diversity of *Theileria equi* and *Babesia caballi* in Mongolian horses. Infection, Genetics and Evolution 119:105571. https://doi.org/10.1016/j.meegid.2024.105571

Pereira MR, Trein CR, Webster A, *et al*., 2023. Comparison of seroprevalence and identification of risk factors for *Theileria equi* in Horses from vector-free and infested areas in southern Brazil. Journal of Equine Veterinary Science 126:104241. https://doi.org/10.1016/j.jevs.2023.104241

Qin S, Kulabieke T, Mizhamuhan D, *et al*., 2025. Molecular prevalence and genotypic diversity of *Theileria equi* in Xinjiang, China, based on three genes. Veterinary Sciences 13:27. https://doi.org/10.3390/vetsci13010027

Ramadan RM, Taha NM, Auda HM, *et al*., 2024. Molecular and immunological studies on *Theileria equi* and its vector in Egypt. Experimental and Applied Acarology 93:439–458. https://doi.org/10.1007/s10493-024-00933-4

Raza A, Ijaz M, Mehmood K, *et al*., 2024. *Theileria equi* infection in working horses of Pakistan: Epidemiology, molecular characterization, and hematobiochemical analysis. Journal of Parasitology 110 (79 – 89). https://doi.org/10.1645/23-58

Soliman AM, Amer MM and Memon FU, 2021. Comparing the efficacy of three diagnostic tools (microscopic examination, conventional PCR, and Real-Time PCR) in detecting *Theileria equi* infection among Egyptian equines. https://doi.org/10.21203/rs.3.rs-770255/v1

Soliman AM, Elhawary NM, Helmy NM, *et al*., 2025. 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. Iranian Journal of Veterinary Research 26(2): 145-151. https://doi.org/10.22099/ijvr.2025.51028.7553

Sumbria D, Singla LD, Kumar S, *et al*., 2016. Spatial distribution, risk factors and haemato-biochemical alterations associated with *Theileria equi* infected equids of Punjab (India) diagnosed by indirect ELISA and nested PCR. Acta Tropica 155:104–112. https://doi.org/10.1016/j.actatropica.2015.12.020

Zajac AM, Conboy GA, Little SE, *et al*., 2021. Veterinary clinical parasitology. Wiley Blackwell: Chichester.