

Epidemiology and risk factors of equine piroplasmosis in game horses across Pakistan

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Received: 21 June 2026 / Revised: 27 September 2026 / Accepted: 08 October 2026 / Published Online: 11 October 2026

Abstract

Equine piroplasmosis (EP), an important tick-borne protozoan disease primarily caused by *Theileria equi* and *Babesia caballi*, persists in healthy carrier horses, compromising equine health, performance and mobility. However, epidemiological data on EP in game horses in Pakistan are sparse. This study investigated the prevalence, geographical distribution, and epidemiological risk factors of *T. equi*, *B. caballi*, and mixed infection in game horses across Pakistan. We conducted a cross-sectional study with 430 horses from 23 districts of Punjab, Sindh, Khyber Pakhtunkhwa, and Gilgit-Baltistan from July 2020 to June 2021. Microscopic examination of blood samples was performed for the presence of piroplasms, and indirect enzyme-linked immunosorbent assays (iELISAs) of sera for antibodies against *T. equi* and *B. caballi* were conducted. We used prevalence estimates, chi-squared or Fisher's exact tests, odds ratios, and exploratory multivariable logistic regression to analyze epidemiological and management data. Microscopic prevalence of *T. equi* and *B. caballi* infection was 26.51% and 0.23%, respectively. Seroprevalence of *T. equi* (33.95%), *B. caballi* (20.23%), and mixed seropositivity (14.88%), as determined by iELISA, was observed. Seroprevalence of *T. equi* differed significantly between provinces, ranging from 43.0% in Punjab to 4.5% in Gilgit-Baltistan. Older horses, especially those >10 years, had a higher likelihood of seropositivity. Prevalence was significantly higher in summer than in winter for both parasites. Ivermectin prophylaxis and acaricide use were associated with lower odds of seropositivity, while body condition score and multispecies farming were associated with variation in the odds of seropositivity. The results indicated a high prevalence of equine piroplasms in game horses across different geographical areas of Pakistan and also revealed significant seasonal, geographical, and management factors influencing infection. Furthermore, the differences between serological and microscopic findings reflect their distinct biological endpoints and highlight the value of complementary diagnostic approaches for assessing exposure and detecting parasitemia. Improving tick management, surveillance, and the strategic use of preventive measures, especially during peak transmission periods, could help reduce transmission and safeguard equine health in Pakistan. Results show there is high exposure to parasites in game horses and highlight the geographical, seasonal and management-related factors associated with seropositivity. However, the present study did not test for active transmission, carrier status, vector competence, or parasite maintenance, and further studies are needed to ascertain the role of game horses in the maintenance of national transmission.

Keywords: Equine piroplasmosis, *Theileria equi*, *Babesia caballi*, Game horses, Risk factors

How to cite this article:

Tayyab MH, Saqib M, Ashfaq K and Sajid MS. Epidemiology and risk factors of equine piroplasmosis in game horses across Pakistan. Asian J. Agric. Biol. 2026; e2026185. DOI: <https://doi.org/10.35495/ajab.2026.185>

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Introduction

Equine piroplasmosis (EP) is an important apicomplexan-induced tick-borne disease of equids caused by the three apicomplexan parasites *Theileria equi*, *Babesia caballi*, and more recently *Theileria haneyi*. These include *T. equi* and *B. caballi*, which are the main organisms studied in epidemiological and diagnostic research of equine piroplasmosis (Cabete et al., 2025). Clinical disease can range from subclinical or chronic infection to acute disease with fever, anemia, icterus, poor performance, and other systemic signs of illness, and both parasites can cause it. Infected equids can be asymptomatic carriers, continuing to spread and persist the parasites within the equine population (Mendoza et al., 2024). *T. haneyi* is a known cause of EP, but this study was limited to *T. equi* and *B. caballi*. This was due to the fact that the study was centered on the two piroplasm species for which the serological assays were well established and used constantly in epidemiological studies, while *T. haneyi* was not included in the serological testing used in the present survey. Hence, the epidemiological estimates reported here apply to *B. caballi* and *T. equi* only and do not necessarily reflect the whole spectrum of the piroplasm infections of horses.

The distribution and activity of competent ixodid ticks are well correlated with the epidemiology of EP. Several tick genera have been involved in the spread of *T. equi* and *B. caballi* (Nadal et al., 2022), and other practices and precautions, such as contaminated instruments, may also contribute to transmission. This long-term carriage of infected equids is significant as evidence of the role of clinically normal animals in maintaining the infection in endemic areas. The distribution and intensity of EP can then be affected by climatic conditions, the abundance of different tick species, animal movements, age, management, and exposure to other livestock (Otgonsuren et al., 2024). EP has significant impacts on veterinary health, the economy, and international trade. Infection can affect athletic performance, working ability, and treatment costs, and can restrict movement and international transportation of horses. Equine movement, breeding and international sporting events, in particular, require detection of carrier animals who may show no clinical signs of infection (Duaso et al., 2025). As a result, EP is reportable and a major constraint on the international movement and trade of horses, with diagnostic testing playing a key role in health

certification and disease-risk management (WOAH, 2025).

EP leads to hematological alterations (Basit et al., 2020) and is difficult to diagnose, especially in chronically infected animals and carriers, in which parasitemia may be low. Intraerythrocytic piroplasms can be seen directly as part of diagnosing high-intensity infections, which is relatively easy because it relies on microscopic examination of stained blood smears; however, it is less sensitive in low infections, especially in carrier animals (Scoles and Ueti, 2015; Wise et al., 2014). *T. equi* can be seen in small pyriform, round, or ovoid bodies, sometimes grouped in the characteristic “Malte cross”, while *B. caballi* is seen as paired pyriform merozoites (Wise et al., 2014; Onyiche et al., 2019). Horses with low or undetectable parasitemia are often tested for antibodies to *T. equi* and *B. caballi* by serological assays, such as competitive and indirect ELISAs. This diagnostic discrepancy has also been seen in Arabian stallions, with Thabet et al. (2024) reporting 30% of the stallions were positive based on microscopy and 39% positive based on cELISA, which again demonstrates the complementary nature of direct parasite detection and serological testing. Still, antibody detection is not a good indicator of circulating parasitemia (Wise et al., 2014; Wise et al., 2013). Molecular tools (e.g., PCR) are used directly to detect parasite DNA and can enhance the ability to detect low numbers of parasites and carrier animals. Molecular assays can complement species-specific testing/confirmation with PCR assays targeting several genomic regions, such as the 18S rRNA gene, the *ema-1* gene of *T. equi*, and *bc48* of *B. caballi* (Wise et al., 2013; Elsayy et al., 2021). Molecular testing was not carried out in the current study as the study resources and diagnostic facilities were dedicated to the microscopic examination and serological testing. Molecular testing offers a more direct method for detecting the presence of parasite DNA and might be more effective for detecting low levels of, or subclinical, infection, but this was not done. Thus the present investigation may be regarded as an epidemiological and serological survey and not as a true picture of the active infection or the amount of the circulating parasitemia. Detectable piroplasm was confirmed by microscopy at the time of sampling and antibody responses were confirmed by indirect ELISA, indicative of previous or current exposure. It is important to take this difference in diagnostic endpoints into account when examining prevalence estimates in this study.

Equine piroplasmosis epidemiology varies widely between countries and regions, depending on tick ecology, climatic zones, horse populations, management systems, and diagnostic methods. A systematic review and meta-analysis provided global estimates of pooled prevalence of *Theileria equi*, *Babesia caballi*, and mixed infections in equids, estimated at 29.4%, 22.3%, and 11.8%, respectively, with significant regional variation in each group (Onyiche et al., 2020).

The prevalence of *T. equi* in Asia was about 18.1% on average, although there are significant reports of higher rates in individual countries and regions. Reports also describe significant exposure to *T. equi* in India: a serological survey of 1,021 equids in three states (Rajasthan, Haryana and Gujarat) showed an overall seroprevalence of 64.44%, with rates of 71.40%, 60.39% and 48.92%, respectively (Dahiya et al., 2018). A serological and molecular study in Punjab, India, revealed that 75% of samples were positive for *T. equi* by cELISA, compared to 14.14% for multiplex PCR, and 1.11% were positive for *B. caballi* by cELISA, with none being positive by PCR, showing the significant differences that can arise based on diagnostic method (Sumbria et al., 2016). Previous studies in Iran have shown a high prevalence of EP (30.07%, 40/133) in horses using PCR in Fars Province and no detection of *B. caballi*. The national mean prevalence rate of *T. equi* was previously estimated at 24.11%, which is lower than the reported prevalence in Fars, and that of *B. caballi* was approximately 5.47% (Kalantari et al., 2022), which is higher. Regional variation is also evident in China, as a large serological survey of 1,990 horses from 10 provinces found *B. caballi* antibodies in 51.16% and *T. equi* in 11.51% of horses, with 7.64% infected with both parasites (Wang et al., 2014). More recent molecular investigation in Jilin Province detected *T. equi* in 27.7% of 220 equids, while *B. caballi* was not detected (Zhao et al., 2020). Significant variation has also been seen outside of Asia. Molecular studies have indicated that 0% of horses in Jordan are infected with *T. equi*, whereas 96.8% in Nicaragua are infected. Serological studies have found very low prevalence in some European countries and extremely high prevalence in South America (Tirosh-Levy et al., 2020).

Pakistan offers an important epidemiological setting to study EP as horses are extensively utilized in the field for transportation, agricultural work, recreation, racing, polo, ceremonies, etc. Earlier research has

shown significant exposure to *T. equi* and *B. caballi* in Pakistani equids. In one of the first large-scale investigations conducted in Punjab, reported seroprevalence rates of 41.2% and 21.6% was for antibodies against *T. equi* and *B. caballi*, respectively, from 430 draught equids, and 10.2% for antibodies against both parasites. The study also showed geographical and host variability in seropositivity and highlighted the need for further epidemiological and molecular studies in Pakistan. Later research found *T. equi* prevalent in equids in Pakistan (Hussain et al., 2014). In another article, an overall seroprevalence of 38.2% was found in 393 horses, donkeys, and mules in Peshawar and Charsadda, Khyber Pakhtunkhwa. Tick infestation, anemia, hematuria, age, and equine species were among the factors associated with seropositivity. Likewise, seasonal fluctuations in *T. equi* seropositivity have been observed in horses in Sargodha, Punjab, indicating that environmental and vector-related factors affect transmission patterns in Pakistan (Afridi et al., 2017). Molecular surveillance in other livestock species also recently showed the presence and epidemiological diversity of piroplasms in Pakistan. In India, Ahmed et al. (2024) detected multiple species of *Theileria* and *Babesia* in cattle from Azad Jammu and Kashmir (AJK) using microscopy and PCR, and Ghani et al. (2024) detected *T. annulata* infection in cattle in Punjab by using microscopy and PCR and established association with tick related and host-management factors. These studies are not on equids but they do show that TBPs and their epidemiological determinants are important in livestock species in Pakistan as well.

However, these studies do not give enough details regarding the epidemiology of EP in game animals of the geographically different regions of Pakistan. Game horses are a unique epidemiological group as they may be managed and/or used very differently from draught or working equines. Racing, polo, riding, tent pegging, exhibitions, ceremonies, and recreational horses can travel from one location to another, be under a variety of husbandry systems, have different types of exposure to livestock and tick-control practices and have contact with horses from other premises. This movement and management can allow for easier contact with infected ticks or allow a greater opportunity for parasite introduction or transmission between otherwise isolated equine populations. Therefore, the epidemiological profiles of *T. equi* and *B. caballi* in game horses may be different from what has been seen in relatively sedentary working or

draught horses, justifying the need for population-specific surveillance. We collected 430 samples from game horses from 23 districts of Punjab, Sindh, Khyber Pakhtunkhwa and Gilgit-Baltistan, Pakistan in the present study. The main goal was to estimate the seroprevalence of antibodies against *T. equi* and *B. caballi* (including mixed seropositivity) by the indirect ELISA. The secondary goal was to assess the prevalence of piroplasms, which can be microscopically detected in blood smears and to compare the microscopic results to serologic results. Geographical, demographic, seasonal, and management-related risk factors for seropositivity and microscopic detection were also assessed. As no PCR was performed, seropositivity was considered to reflect past or present exposure and not necessarily active infection, and microscopic positivity was considered to indicate the presence of piroplasms at the time of sampling. Therefore, a sero-epidemiological cross-sectional survey of piroplasmosis in game horses of Pakistan was designed.

Material and Methods

Study design and study locales

We investigated *Theileria equi* and *Babesia caballi* infection in game horses in Pakistan through a cross-sectional epidemiological study conducted from July 2020 to June 2021. Blood samples were obtained from 430 horses from 23 selected districts across four provinces/administrative regions of Punjab, Sindh, Khyber Pakhtunkhwa (KPK) and Gilgit-Baltistan. Districts were chosen based on the distribution of the equine population, and sampling within districts was carried out in a samples-available and proportionate manner. A formal sampling frame of the game horse

population and a sample denominators was not possible because there was no reliable or official register of game horses in the study areas. The game horses were mainly kept on farms or facilities, usually more than three on each farm/ facility, and in some of the larger facilities as many as 50 or more horses were kept. Horses also might be sent to districts from other parts of the country, or other cities for sports and other purposes. Thus, accessible population was defined as facilities and events population instead of an enumerated population of district. The distribution and availability of game horses were used as a basis for selecting districts and sampling was done within districts by an available and proportionate sampling method with relatively larger facilities contributing proportionately more game horses. The final sample of 430 horses was therefore selected pragmatically in order to cover as widely as possible the selected Districts and to ensure that the sample size was broadly representative of the available game-horse population, and not as a formal national probability sample. The study was not equally allocated by sex or equal allocation of samples among provinces. Final sex distribution was 230 males (53.5%) and 200 female horses (46.5%). The slight difference in the number of male and female horses resulted from the availability of eligible game horses during field sampling, and no attempt was made to force an equal 1:1 sex distribution. The distribution of samples was 200 from Punjab, 94 from Sindh, 92 from Khyber Pakhtunkhwa and 44 from Gilgit-Baltistan ($n = 430$). Districts were chosen based on the distribution of the equine population and sampling within the selected districts was based on a sample available and proportionate rather than an equal allocation of samples between provinces or between males and females.

Table-1. Distribution of sampled game horses among the four provinces/administrative regions of Pakistan.

Province/administrative region	No. sampled	Percentage
Punjab	200	46.5
Sindh	94	21.9
Khyber Pakhtunkhwa	92	21.4
Gilgit-Baltistan	44	10.2
Total	430	100.0

Due to the fact that game horses were kept on farms or facilities more than one horse was sampled in the same facility. Horses were sampled proportionately to the number of horses in the larger facilities, leading to a higher number of horses being sampled in those facilities as opposed to smaller facilities like a polo club. However, not all sampled horses had a complete facility-level sampling frame, and facility identifiers were not consistent; so the number of horses sampled in each individual facility could not be accurately reconstructed post hoc. Thus, adjustment of within-facility clustering was not possible. The analyses of the statistical data, therefore, should be viewed as exploratory and potentially non-independence of observations within facilities.

Epidemiological data

A structured questionnaire was used for data collection, both at the level of the animal and the management level, and the data gathered were the age, sex, breed, body condition score (BCS), clinical history, previous treatment, tick control and preventative measures, travel records, herd size, reason for keeping horses and contact with other species of animals. Farm managers/owners rather than attending veterinarians were the major sources of information on prevention measures. Hence, the treatment exposure was documented primarily by the active ingredient used (such as ivermectin, imidocarb, or acaricide) and not necessarily by the exact dose, frequency, or duration of treatment and not necessarily by the recency or exact timing of the treatment. The following variables were subsequently grouped and categorized for epidemiological analysis: treatment use/non-use, reported treatment use/non-use.

Collection and processing of blood

Blood was aseptically collected from the jugular vein of each horse (approx. 8 mL). About 5 mL was placed in tubes containing no additives for serological and biochemical analysis, and about 3 mL was collected in tubes containing EDTA for blood-smear preparation. Samples were sent to the Laboratory of Veterinary Preventive Medicine and Public Health, University of Agriculture, Faisalabad, Pakistan under refrigerated conditions. Blood was centrifuged and serum stored for serological examination.

Microscopic examination

Blood smears were prepared from EDTA-anticoagulated blood, fixed in absolute methanol, air-dried, and stained with rapid Romanowsky stain (RAL DIFF-QUIK™, TCS Biosciences, UK) strictly following the manufacturer's instructions. The smears were stained and examined under oil immersion at 100× magnification to identify and morphologically confirm piroplasms, consistent with *T. equi* and *B. caballi*.

Serological examination

We used commercial indirect ELISA kits (Biostone Animal Health, USA: *T. equi* and *Babesia caballi*) to detect serum antibodies to *T. equi* and *B. caballi*. These assays detect antibody levels to *T. equi* and *B. caballi*. All serum samples were diluted 1:20 and tested according to the manufacturer's instructions. After incubation and washing, HRP-labeled antibody and TMB substrate were added, and the reaction was terminated before OD determination at 450nm. According to the manufacturer's interpretation criteria, samples with percent positivity (PP) ≥60% were classified as positive, whereas samples with PP <60% were classified as negative.

Statistical analysis

The epidemiological survey data were recorded in an electronic spreadsheet (Microsoft Excel) and then analyzed statistically. We determined prevalence by dividing the number of horses diagnosed positive by the number of horses tested, then multiplying by 100 to obtain a percentage. We calculated exact 95% confidence intervals (95% CIs) for prevalence estimates. We examined initial associations between infection status and categorical epidemiological variables using contingency-table analysis, including province, sex, age group, purpose of horse keeping, herd size, multi-species farming, traveling frequency, body condition score, imidocarb prophylaxis, and ivermectin prophylaxis. We used an appropriate reference category for each categorical variable to calculate odds ratios (OR) and 95% confidence intervals (CIs). For statistical associations, Pearson's chi-square (χ^2) test was used, and Fisher's exact test was used when expected cell frequencies were small. We used $P < 0.05$ as the level of statistical significance.

Variables showing indications of association in the univariable analysis were carried forward for

exploratory multivariable analysis. We assessed the probability of infection using logistic regression to determine whether epidemiological variables contributed to this probability independently of other factors. We adjusted all odds ratios (ORs) and calculated 95% confidence intervals (CIs) to describe the direction and magnitude of associations. We prioritized variables associated at the univariable level, while still including biologically relevant variables where appropriate. We did not include all epidemiological variables in the same model because of potential multicollinearity. We aggregated the data at the category level, not the horse level; the multivariable estimates presented in the results were exploratory approximations, not direct estimates of the variables of interest, which could be regarded as individual-level regression coefficients. The multivariable results were presented interpretively and were intended to identify the most likely factors associated with infection. Since full facility identifiers were not available, clustering by facility could not be included in the statistical models, and associations and P values reported as exploratory, with possible within-facility correlation noted as a limitation.

Results

Blood smear examination of the game horses revealed piroplasm as the predominant finding of *Theileria equi* in Pakistan. Out of 430 horses, 114 were positive for *T. equi*, resulting in an overall microscopic prevalence of 26.51% (95% CI: 22.45–31.00%). The association between microscopic detection status and parasite species was statistically significant ($\chi^2 = 189.23$, $df = 2$, $p < 0.0001$). By contrast, only one of 430 horses (0.23% [0.01 to 1.49%]) was found to be *Babesia caballi* positive microscopically. Mixed infection with *T. equi* and *B. caballi* was also detected in 1 of 430 horses (0.23%; 95% CI: 0.01–1.49%). Therefore, microscopic examination revealed a significantly greater prevalence of *T. equi* than of *B. caballi* or mixed infection in the spotted horses analyzed.

The serological method (indirect ELISA) detected a higher proportion of antibody-positive horses than microscopic examination detected piroplasms. Overall, 146 of 430 horses were seropositive for *T. equi*, giving a seroprevalence of 33.95% (95% CI: 29.50–38.70%). Serological detection categories were significantly associated ($\chi^2 = 28.64$, $df = 2$, $p < 0.0001$). The seroprevalence of *Babesia caballi* antibodies was 20.23% (95% CI: 16.60–24.41%) in 87 of 430 horses.

The reported odds ratio for *B. caballi* seropositivity was 1.36 (95% CI: 0.96–1.93). Importantly, 64 of 430 horses (14.88%) were seropositive for mixed infection with *T. equi* and *B. caballi* (95% CI: 11.72–18.68%). Hence, iELISA identified a higher proportion of horses with antibodies to *T. equi*, *B. caballi*, and both parasites than were positive by microscopy. These findings should be interpreted in relation to the different biological endpoints measured by two methods, with serology indicating exposure or immune response and microscopy indicating detectable circulating piroplasms at the time of sampling. The results of the microscopic and serological examinations showed a significant discrepancy, especially for *B. caballi*. In contrast, only one of 430 horses (0.23%) had *B. caballi* detected in its blood under the microscope whereas 87 of 430 horses (20.23%) were seropositive using iELISA. In a similar way, mixed infection was only identified in one horse (0.23%) by microscopic examination while 64 horses (14.88%) were mixed seropositive by iELISA. This discrepancy may be due to the detection of intraerythrocytic parasites in the micro examination and the iELISA to antibodies which suggest past or current exposure to the parasite, which may be less sensitive if parasitemia is low, especially in chronically infected or carrier animals. Hence, this seroprevalence should not be taken to mean that there was a similar prevalence of active parasitemia observed by iELISA. Both diagnostic methods assess different biological endpoints and, therefore, should be interpreted appropriately.

Seroprevalence of *Theileria equi*

The overall prevalence of *Theileria equi* was 34.0% (146/430). Prevalence varied significantly between provinces ($\chi^2 = 26.21$, $P < 0.001$). Punjab showed the highest prevalence (43.0%; 95% CI: 36.0–50.2%), whereas Gilgit Baltistan had the lowest prevalence (4.5%; 95% CI: 0.6–15.5%). Compared with Punjab, horses from KPK (OR = 0.50, 95% CI: 0.29–0.85) and Gilgit Baltistan (OR = 0.07, 95% CI: 0.02–0.27) had significantly lower odds of infection. The geographical distribution of *T. equi*, *B. caballi*, and mixed infections among game horses in Pakistan varied widely across districts. In the case of *T. equi*, 72.22% of animals were seropositive in D.I Khan, followed by Lahore, Faisalabad and Abbottabad, with very low or none in several districts of Gilgit-Baltistan and some districts of Punjab (Figure 1). Overall, however, the difference in seroprevalence among

districts was not significantly different ($\chi^2 = 17.25$, $df = 18$, $p = 0.506$).

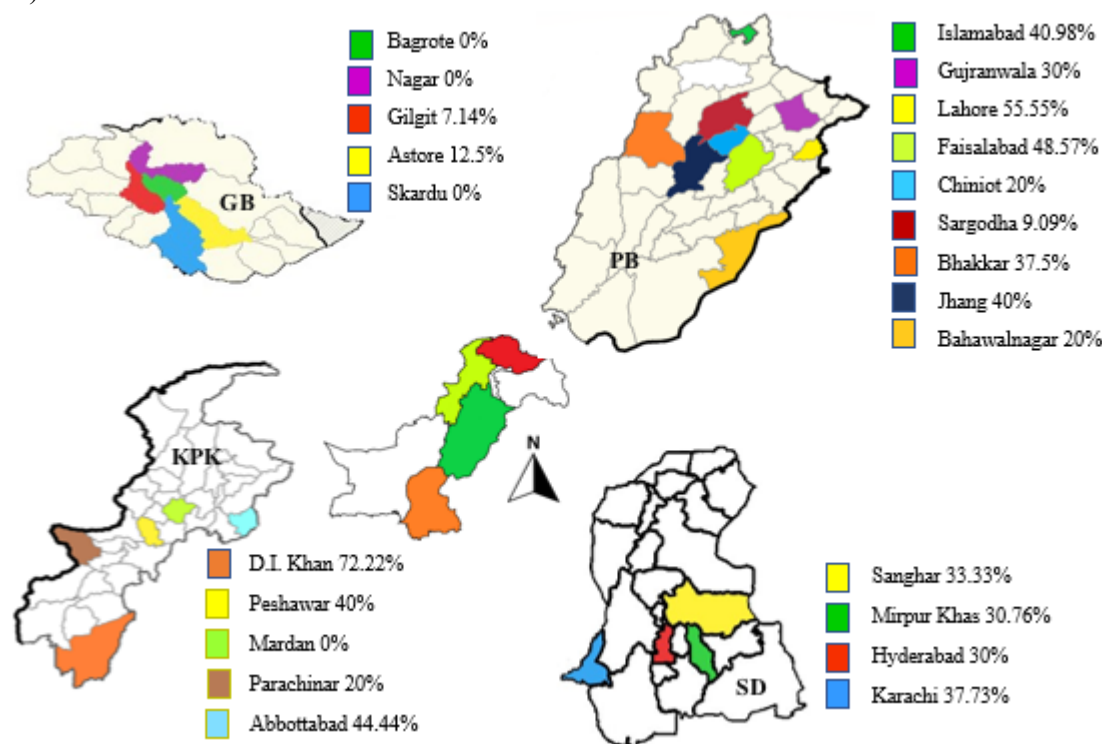


Figure-1. Seroprevalence of *Theileria equi* in different districts of Pakistan.

There was no significant difference in seropositivity for *T. equi* between males and females ($\chi^2 = 0.08$, $P = 0.774$). The prevalence was 34.8% (95% CI: 28.7–41.3%) in males and 33.0% (95% CI: 26.5–40.0%) in females, with females showing similar odds of infection (OR = 0.92, 95% CI: 0.62–1.38) (Table 2). Infection was significantly related to age ($\chi^2 = 22.90$, $P < 0.001$). Horses older than 10 years exhibited the highest prevalence (56.8%; 95% CI: 45.8–67.4%), whereas animals aged 5–10 years (OR = 0.33, 95% CI: 0.20–0.57) and younger than 5 years (OR = 0.26, 95% CI: 0.15–0.47) had significantly lower odds of infection. There was no significant association between BCS and infection ($\chi^2 = 4.48$, $P = 0.106$); however, when a horse had a BCS >5 , they were less likely to be infected than horses with a BCS ≤ 3 —season strongly influenced seropositivity ($\chi^2 = 45.57$, $P < 0.001$). Prevalence was significantly higher in horses sampled in summer (45.7%) than in winter (6.9%), and winter sampling had substantially lower

odds of infection (OR = 0.09, 95% CI: 0.05–0.19). Ivermectin prophylaxis was significantly associated with reduced infection ($\chi^2 = 73.74$, $P < 0.001$). Infection prevalence was 15.4% in horses treated with ivermectin, compared with 62.4% in untreated horses (OR = 0.11, 95% CI: 0.07–0.18). The same correlation was found for acaricide use, as the data were the same. In these pooled results, there was no meaningful difference in infection with imidocarb prophylaxis, but the overall chi-square statistic was significant. The prevalence was 34.5% in untreated horses and 32.9% in treated horses (OR = 0.93, 95% CI: 0.60–1.44). Frequent traveling ($\chi^2 = 11.44$, $P < 0.001$), herd size ($\chi^2 = 11.77$, $P = 0.003$), and the presence of multi-species farming ($\chi^2 = 36.88$, $P < 0.001$) were significantly associated with *T. equi* infection. Horses kept on multi-species farms had almost three times the risk of infection when compared to those on single-species farms.

Table-2. Univariable analysis of factors associated with *Theileria equi* infection in game horses.

Variable	Category	Positive/Tested	Prevalence % (95% CI)	OR (95% CI)	χ^2	P-value
Province	Punjab (Reference)	86/200	43.0 (36.0–50.2)	1.00	26.21	<0.001
	Sindh	33/94	35.1 (25.5–45.7)	0.72 (0.43–1.19)		
	KPK	25/92	27.2 (18.4–37.6)	0.50 (0.29–0.85)		
	Gilgit Baltistan	2/44	4.5 (0.6–15.5)	0.07 (0.02–0.27)		
Sex	Male (Reference)	80/230	34.8 (28.7–41.3)	1.00	0.08	0.774
	Female	66/200	33.0 (26.5–40.0)	0.92 (0.62–1.38)		
Age group	>10 years (Reference)	50/88	56.8 (45.8–67.4)	1.00	22.90	<0.001
	>5–10 years	56/187	29.9 (23.4–37.0)	0.33 (0.20–0.57)		
	<5 years	40/155	25.8 (19.1–33.4)	0.26 (0.15–0.47)		
Body condition score	≤3 (Reference)	13/25	52.0 (31.3–72.2)	1.00	4.48	0.106
	>3–≤5	113/325	34.8 (29.7–40.2)	0.49 (0.22–1.08)		
	>5	20/80	25.0 (16.0–36.1)	0.31 (0.12–0.80)		
Season	Summer (Reference)	137/300	45.7 (40.0–51.5)	1.00	45.57	<0.001
	Winter	9/130	6.9 (3.2–12.8)	0.09 (0.05–0.19)		
Ivermectin prophylaxis	No (Reference)	106/170	62.4 (54.6–69.7)	1.00	73.74	<0.001
	Yes	40/260	15.4 (11.2–20.5)	0.11 (0.07–0.18)		
Imidocarb prophylaxis	No (Reference)	100/290	34.5 (29.0–40.3)	1.00	14.89	<0.001
	Yes	46/140	32.9 (25.2–41.3)	0.93 (0.60–1.44)		
Use of acaricides	No (Reference)	106/170	62.4 (54.6–69.7)	1.00	73.74	<0.001
	Yes	40/260	15.4 (11.2–20.5)	0.11 (0.07–0.18)		
Frequent travelling	Yes (Reference)	132/360	36.7 (31.7–41.9)	1.00	11.44	<0.001
	No	14/70	20.0 (11.4–31.3)	0.43 (0.23–0.82)		
Herd size	11–50 (Reference)	82/200	41.0	1.00	11.77	0.003

Variable	Category	Positive/Tested	Prevalence % (95% CI)	OR (95% CI)	χ^2	P-value
			(34.1–48.2)			
	>50	47/160	29.4 (22.5–37.1)	0.60 (0.38–0.95)		
	<10	17/70	24.3 (14.8–36.0)	0.46 (0.24–0.88)		
Purpose	Recreation/Dancing (Reference)	25/50	50.0 (35.5–64.5)	1.00	7.44	0.190
	Others/Pet	11/30	36.7 (19.9–56.1)	0.58 (0.22–1.50)		
	Ceremony/Exhibition	14/40	35.0 (20.6–51.7)	0.54 (0.23–1.26)		
	Tent pegging	20/60	33.3 (21.7–46.7)	0.50 (0.23–1.08)		
	Racing/Riding	38/120	31.7 (23.5–40.9)	0.46 (0.23–0.91)		
	Polo	38/130	29.2 (21.6–37.8)	0.41 (0.21–0.81)		
Multi-species farm	Yes (Reference)	121/270	44.8 (38.8–50.9)	1.00	36.88	<0.001
	No	25/160	15.6 (10.4–22.1)	0.24 (0.15–0.37)		

Multivariable analysis of risk factors associated with *Theileria equi* seropositivity

To explore the factors that were associated with *Theileria equi* seropositivity, a reconstructed multivariable logistic regression analysis was performed (Table 3). The analysis was based on aggregated epidemiological data and as such, the adjusted estimates are intended as exploratory reconstructed approximations and should not be interpreted as conventional individual-level adjusted regression coefficients. Thus the reconstructed analysis was descriptive and was used to describe the direction and approximate magnitude of association between the available epidemiological variables, and not to define independent causal effects.

Horses in Gilgit-Baltistan were less likely to be seropositive for *T. equi* (reconstructed AOR = 0.09, 95% CI: 0.02–0.42; P = 0.002) than horses in Punjab. Similarly, the estimated odds of horses from Khyber Pakhtunkhwa were also slightly lower (AOR = 0.58, 95% CI: 0.32–1.05) but was not significant after adjustment. The association for Sindh was similarly attenuated (AOR = 0.78, 95% CI: 0.45–1.35).

Horses older than 10 years had higher estimated odds of *T. equi* seropositivity than horses younger than 5

years (AOR = 2.34, 95% CI: 1.29–4.25; P = 0.005), whereas horses aged 5–10 years did not differ significantly from the youngest age group (AOR = 1.27, 95% CI: 0.76–2.12; P = 0.36). Increasing odds of seropositivity were estimated for horses with a BCS ≤ 3 compared with horses with a BCS > 5 (AOR = 2.54, 95% CI: 1.04–6.22; P = 0.041).

The reconstructed analysis showed a strong association between *T. equi* seropositivity and season. Results indicated that horses sampled in summer were more likely to be seropositive than horses sampled in winter (AOR = 4.02, 95% CI: 1.90–8.49; P < 0.001). The absence of ivermectin prophylaxis was also a risk factor for seropositivity (AOR = 4.31, 95% CI: 2.57–7.23; P < 0.001). Since these estimates were derived using aggregated data, they should not be taken as proof of a causal reduction in infection from ivermectin prophylaxis.

After adjustment, associations of frequent traveling and herd size were weaker. The association between traveling horses and increased odds of infection was not statistically significant (AOR = 1.72, 95% CI: 0.88–3.36, P = 0.11). A similar association was observed for horses kept in herds of 11–50 animals, with slightly increased odds compared with horses in herds of fewer than 10 animals, but this was not

statistically significant (AOR = 1.72, 95% CI: 0.87–3.41; $P = 0.12$). Purpose of horse keeping was not independently associated with infection. Odds were lower than for recreation /dancing horses in most classes, with a near-significant association for polo (AOR = 0.52, 95% CI: 0.27–0.99; $P = 0.047$). Multispecies farming was still an important independent epidemiological factor. Horses raised on multiple species farms were about 2.7 times more

likely to be infected with *T. equi* than those raised on a single species farm (AOR = 2.73, 95% CI: 1.55–4.80; $P < 0.001$). The original data showed 121/270 positive horses on multi-species farms versus 25/160 on single-species farms. When we accounted for other epidemiological characteristics, summer season, lack of ivermectin prophylaxis, multi-species farming, age, and body condition were the main factors potentially associated with *T. equi* infection overall.

Table-3. Reconstructed exploratory multivariable analysis of factors associated with *Theileria equi* seropositivity.

Variable	Category	Adjusted OR	Approx. 95% CI	Approx. P-value
Province	Punjab (Reference)	1.00	—	—
	Sindh	0.78	0.45–1.35	0.37
	KPK	0.58	0.32–1.05	0.071
	Gilgit Baltistan	0.09	0.02–0.42	0.002
Age group	<5 years (Reference)	1.00	—	—
	5–10 years	1.27	0.76–2.12	0.36
	>10 years	2.34	1.29–4.25	0.005
Body condition score	>5 (Reference)	1.00	—	—
	>3–≤5	1.48	0.82–2.67	0.19
	≤3	2.54	1.04–6.22	0.041
Season	Winter (Reference)	1.00	—	—
	Summer	4.02	1.90–8.49	<0.001
Ivermectin prophylaxis	Yes (Reference)	1.00	—	—
	No	4.31	2.57–7.23	<0.001
Frequent travelling	No (Reference)	1.00	—	—
	Yes	1.72	0.88–3.36	0.11
Herd size	<10 (Reference)	1.00	—	—
	11–50	1.72	0.87–3.41	0.12
	>50	1.19	0.57–2.49	0.64
Purpose	Recreation/Dancing (Reference)	1.00	—	—
	Others/Pet	0.70	0.28–1.76	0.46
	Ceremony/Exhibition	0.67	0.29–1.55	0.35
	Tent pegging	0.63	0.30–1.31	0.22
	Racing/Riding	0.59	0.31–1.12	0.11
	Polo	0.52	0.27–0.99	0.047
Multi-species farm	No (Reference)	1.00	—	—
	Yes	2.73	1.55–4.80	<0.001

Note: Aggregated epidemiological data were used to reconstruct analyses to obtain adjusted odds ratios (AORs). Thus, these estimates are tentative approximations and should not be taken as typical regression coefficients with individual variation eliminated or as proof of causal effects.

Seroprevalence of *Babesia caballi*

There was a significant difference in the seroprevalence of *Babesia caballi* between provinces ($\chi^2 = 17.36$, $P < 0.001$). The highest prevalence was recorded in Sindh (29.8%; 95% CI: 21.5–39.7%), which served as the reference category, followed by Khyber Pakhtunkhwa (26.1%; 95% CI: 18.2–35.9%). In contrast, Punjab exhibited a significantly lower prevalence (17.0%; 95% CI: 12.4–22.8%; OR = 0.48, 95% CI: 0.27–0.86), while Gilgit-Baltistan showed the

lowest prevalence (2.3%; 95% CI: 0.4–11.8%; OR = 0.05, 95% CI: 0.01–0.42). The same type of geographic distribution was seen with *B. caballi*. D.I. Khan had the highest prevalent substantial (55.55%) followed by Peshawar and some districts of Sindh while no seropositive animals were found in some districts especially in Gilgit-Baltistan and some parts of Punjab (Figure 2). However, there was no significance difference between the districts ($\chi^2 = 17.36$, $df = 17$, $p = 0.4299$).

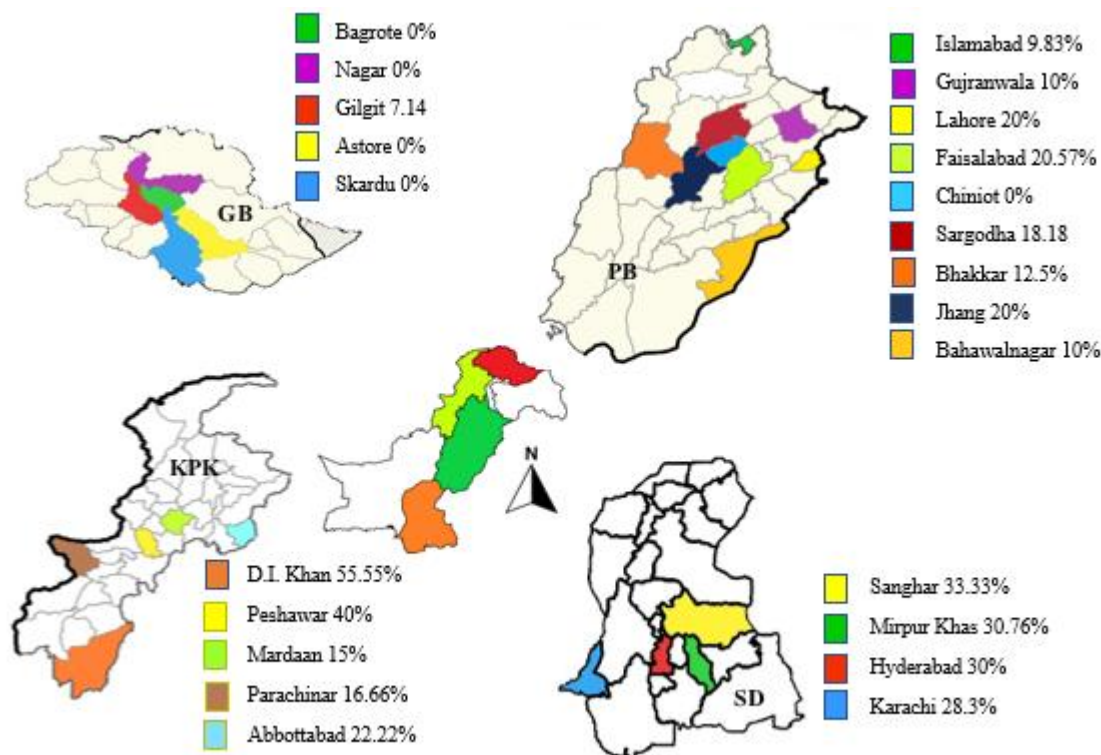


Figure-2. Seroprevalence of *Babesia caballi* in different districts of Pakistan.

There was no significant difference between the prevalence of seropositivity of *B. caballi* in males and females ($\chi^2 = 0.00$; $P = 1.000$). Male horses exhibited a slightly higher seroprevalence (20.4%; 95% CI: 15.7–26.1%) than females (20.0%; 95% CI: 15.0–26.1%), with comparable odds of infection (OR = 0.97, 95% CI: 0.61–1.56) (Table 4). We found a significant association between *B. caballi* infection and age ($\chi^2 = 12.33$, $P = 0.002$). The highest seroprevalence (28.4% (CI: 21.9–35.9%)) was found among horses older than 10 years, which was used as the reference group. Horses aged 5–10 years had a significantly lower prevalence (18.2%; 95% CI: 13.3–24.3%; OR = 0.56, 95% CI: 0.34–0.93), while animals

younger than 5 years showed the lowest prevalence (10.2%; 95% CI: 5.5–18.3%; OR = 0.29, 95% CI: 0.13–0.62). No significant association of *B. caballi* seropositivity was determined with breed ($\chi^2 = 4.46$, $P = 0.615$). The prevalence was highest in indigenous horses (24.1%; 95% CI: 18.3–31.1%), followed by Sian (22.2%) and Arab (20.0%) horses, and comparatively low in Thoroughbred (16.8%), Anmol (16.7%), Suffolk (12.5%), and Pony (0.0%). None of the above differences were statistically significant. There was a strong association between seropositivity for *B. caballi* and body condition score (BCS) ($\chi^2 = 31.61$, $P < 0.001$). Horses with a BCS ≤ 3 had the highest prevalence (64.0%; 95% CI: 44.5–79.8) and

were used as the reference group. Horses with a BCS of >3 to ≤ 5 (17.8%; 95% CI: 14.1–22.4%; OR = 0.12, 95% CI: 0.05–0.29) and those with a BCS >5 (16.2%; 95% CI: 9.7–25.8%; OR = 0.11, 95% CI: 0.04–0.30) had significantly lower odds of infection. The season significantly affected the prevalence of *B. caballi* infection ($\chi^2 = 21.65$, $P < 0.001$). Horses sampled in the summer had a significantly higher proportion of seropositive horses (26.3% of horses, 95% CI: 21.7–31.6%) than in the winter (6.2% of horses, 95% CI: 3.2–11.7%), with winter horses having lower odds of seropositivity (OR = 0.18, 95% CI: 0.09–0.39). Management practices were strongly associated with seropositivity for *B. caballi*. Horses that did not receive ivermectin tick prophylaxis had a significantly higher prevalence (32.4%; 95% CI: 25.8–39.7%) than treated horses (12.3%; 95% CI: 8.9–16.9%), and ivermectin treatment was associated with approximately 71% lower odds of *B. caballi* seropositivity (OR = 0.29, $P < 0.001$). Similarly, horses that did not receive imidocarb prophylaxis exhibited a higher prevalence (24.8%; 95% CI: 20.2–30.1%) than treated horses (10.7%; 95% CI: 6.6–16.9%), corresponding to significantly reduced odds among treated animals (OR = 0.36, 95% CI: 0.20–0.66; $\chi^2 = 10.79$, $P = 0.001$). Likewise, the absence of acaricide use was associated with a significantly higher prevalence (32.4%; 95% CI: 25.8–39.7%) compared with acaricide-treated horses (12.3%; 95% CI: 8.9–

16.9%), with acaricide use significantly associated with lower odds of *B. caballi* seropositivity (OR = 0.29, 95% CI: 0.18–0.48; $\chi^2 = 24.36$, $P < 0.001$).

There was no significant association between frequent traveling and *B. caballi* seropositivity ($\chi^2 = 3.39$, $P = 0.066$), with horses that were frequent travelers producing a higher prevalence of seropositivity (21.9%; 95% CI: 18.0–26.5%) than non-travelers (11.4%; 95% CI: 5.9–21.0%). Similarly, there was no statistically significant association between infection and herd size ($\chi^2 = 0.87$, $P = 0.646$). Still, most infections (22.0%) occurred in herds of 11–50 animals, followed by herds of more than 50 animals (19.4%) and fewer than 10 animals (17.1%). The overall function of horses did not significantly correlate with *B. caballi* seropositivity ($\chi^2 = 5.49$, $P = 0.359$). The highest incidence (30.0%) was seen in horses that were kept as pets or for other affiliations, followed by recreation/dancing (26.0%), ceremony/exhibition (25.0%), polo (19.2%), racing/riding (18.3%), and tent pegging (13.3%). In contrast, infection was significantly associated with the presence of a multi-species farm ($\chi^2 = 19.70$, $P < 0.001$). The prevalence was significantly higher on multi-species farms (27.0%, 95% CI: 22.1–32.6%) than on single-species farms (8.8%, 95% CI: 5.3–14.2%), and the odds of infection were significantly lower for horses on single-species farms (OR = 0.26, 95% CI: 0.14–0.48).

Table-4. Univariable analysis of factors associated with *Babesia caballi* infection in game horses.

Variable	Category	Positive/Tested	Prevalence % (95% CI)	OR (95% CI)	χ^2	P-value
Province	Sindh (Reference)	28/94	29.8 (21.5–39.7)	Reference	17.36	<0.001
	KPK	24/92	26.1 (18.2–35.9)	0.83 (0.44–1.58)		
	Punjab	34/200	17.0 (12.4–22.8)	0.48 (0.27–0.86)		
	Gilgit Baltistan	1/44	2.3 (0.4–11.8)	0.05 (0.01–0.42)		
Sex	Male (Reference)	47/230	20.4 (15.7–26.1)	Reference	0.00	1.000
	Female	40/200	20.0 (15.0–26.1)	0.97 (0.61–1.56)		
Age group	>10 years (Reference)	44/155	28.4 (21.9–35.9)	Reference	12.33	0.002
	>5–10 years	34/187	18.2 (13.3–24.3)	0.56 (0.34–0.93)		
	<5 years	9/88	10.2 (5.5–18.3)	0.29 (0.13–0.62)		
Breed	Indigenous (Reference)	41/170	24.1 (18.3–31.1)	Reference	4.46	0.615

Variable	Category	Positive/Tested	Prevalence % (95% CI)	OR (95% CI)	χ^2	P-value
Body score	Siaen	10/45	22.2 (12.5–36.3)	0.90 (0.41–1.97)	31.61	<0.001
	Arab	8/40	20.0 (10.5–34.8)	0.79 (0.34–1.84)		
	Thoroughbred	25/149	16.8 (11.6–23.6)	0.63 (0.36–1.11)		
	Anmol	1/6	16.7 (3.0–56.4)	0.63 (0.07–5.54)		
	Suffolk	2/16	12.5 (3.5–36.0)	0.45 (0.10–2.06)		
	Pony	0/4	0.0 (0.0–49.0)	0.35 (0.02–6.58)		
	condition ≤ 3 (Reference)	16/25	64.0 (44.5–79.8)	Reference		
	$>3-\leq 5$	58/325	17.8 (14.1–22.4)	0.12 (0.05–0.29)		
	>5	13/80	16.2 (9.7–25.8)	0.11 (0.04–0.30)		
	Season					
Season	Summer (Reference)	79/300	26.3 (21.7–31.6)	Reference	21.65	<0.001
	Winter	8/130	6.2 (3.2–11.7)	0.18 (0.09–0.39)		
Ivermectin prophylaxis	No (Reference)	55/170	32.4 (25.8–39.7)	Reference	24.36	<0.001
	Yes	32/260	12.3 (8.9–16.9)	0.29 (0.18–0.48)		
Imidocarb prophylaxis	No (Reference)	72/290	24.8 (20.2–30.1)	Reference	10.79	0.001
	Yes	15/140	10.7 (6.6–16.9)	0.36 (0.20–0.66)		
Use of acaricides	No (Reference)	55/170	32.4 (25.8–39.7)	Reference	24.36	<0.001
	Yes	32/260	12.3 (8.9–16.9)	0.29 (0.18–0.48)		
Frequent travelling	Yes (Reference)	79/360	21.9 (18.0–26.5)	Reference	3.39	0.066
	No	8/70	11.4 (5.9–21.0)	0.46 (0.21–1.00)		
Heads per herd	11–50 (Reference)	44/200	22.0 (16.8–28.2)	Reference	0.87	0.646
	>50	31/160	19.4 (14.0–26.2)	0.85 (0.51–1.43)		
	<10	12/70	17.1 (10.1–27.6)	0.73 (0.36–1.49)		
Purpose	Others/Pet/Affiliations (Reference)	9/30	30.0 (16.7–47.9)	Reference	5.49	0.359
	Recreation/Dancing	13/50	26.0 (15.9–39.6)	0.82 (0.30–2.24)		
	Ceremony/Exhibition	10/40	25.0 (14.2–40.2)	0.78 (0.27–2.24)		

Variable	Category	Positive/Tested	Prevalence % (95% CI)	OR (95% CI)	χ^2	P-value
Multi-species farm	Polo	25/130	19.2 (13.4–26.8)	0.56 (0.23–1.36)	19.70	<0.001
	Racing/Riding	22/120	18.3 (12.4–26.2)	0.52 (0.21–1.30)		
	Tent pegging	8/60	13.3 (6.9–24.2)	0.36 (0.12–1.06)		
	Yes (Reference)	73/270	27.0 (22.1–32.6)	Reference		
	No	14/160	8.8 (5.3–14.2)	0.26 (0.14–0.48)		

Multivariable analysis of risk factors associated with *Babesia caballi* infection

A model of the major epidemiological factors potentially associated with *Babesia caballi* infection was reconstructed as a multivariable logistic regression model to investigate the independent role of these factors. The variables that appeared to be associated in the univariable analysis were considered for the reconstructed model. Adjusted estimates should be interpreted as approximate and not as directly fitted regression coefficients as the original individual level data was not available. When the other factors in the model were controlled for, province still was a significant factor influencing *B. caballi* seropositivity. The odds of infection in Sindh horses were found to be about 5.8 times higher than the Gilgit Baltistan horses (reconstructed AOR = 5.80; 95% CI: 1.10–31.00; $P = 0.039$). Horses from Khyber Pakhtunkhwa also had higher odds (AOR = 5.10; 95% CI: 0.95–27.40) but the Association was marginally non-significant ($P = 0.057$; however, the odds for Punjab were lower and non-significant after adjustment (AOR = 3.60; 95% CI: 0.72–18.00; $P = 0.116$).

Age remained a risk factor for infection, with older horses at higher risk. Older age (in years) was associated with a 2.3-fold increased risk of *B. caballi* infection (AOR = 2.30; 95% CI 1.00–5.40; $P = 0.049$) in animals older than 10 years compared with those under 5 years of age. Horses aged 5–10 years showed a smaller and non-significant increase in odds (AOR = 1.50; 95% CI: 0.65–3.50; $P = 0.33$). Body condition score also remained an important factor. Horses with a BCS ≤ 3 had approximately threefold greater odds of infection than horses with a BCS > 5 (AOR = 3.10;

95% CI: 1.05–9.20; $P = 0.040$). Animals in the intermediate body condition ($>3 \leq 5$) did not differ significantly from the reference group. Season showed the strongest association in the reconstructed model. Horses checked in the summer had about three times higher odds of *B. caballi* infection compared to those checked in the winter (AOR = 3.10, 95% CI: 1.45–6.60; $P = 0.003$). This result is similar to the significantly higher prevalence found in the univariable analysis in summer. After adjustment, lack of ivermectin prophylaxis remained significantly associated with infection. Horses that were not treated with ivermectin had nearly twice the odds of *B. caballi* infection as horses that were treated with ivermectin to prevent infection (AOR = 2.05; 95% CI: 1.18–3.55; $P = 0.011$). The association with absence of imidocarb prophylaxis was somewhat weakened after adjustment and was not statistically significant (AOR = 1.65; 95% CI: 0.88–3.10; $P = 0.116$). Traveling trips were also not independently associated with infection after adjustment (AOR = 1.45; 95% CI: 0.68–3.10; $P = 0.33$). In contrast, multi-species farming remained an important epidemiological factor. The odds of infection in horses raised on multi-species farms were about 2.4 times higher than those on single-species farms (AOR = 2.35; 95% CI: 1.25–4.42; $P = 0.008$). The reconstructed model indicated that season of the year, body condition, absence of ivermectin prophylaxis, farming multiple species, age, and geographical location were the key factors potentially associated with *B. caballi* infection. The following adjusted estimates should, however, be considered with some caution, as they are reconstructed from aggregated marginal data and not estimated from the individual-horse observations.

Table-5. Reconstructed exploratory multivariable analysis of factors associated with *Babesia caballi* seropositivity.

Variable	Category	Reconstructed AOR	Approx. 95% CI	Approx. P-value
Province	Gilgit Baltistan (Reference)	1.00	—	—
	Sindh	5.80	1.10–31.00	0.039
	KPK	5.10	0.95–27.40	0.057
	Punjab	3.60	0.72–18.00	0.116
Age group	<5 years (Reference)	1.00	—	—
	5–10 years	1.50	0.65–3.50	0.33
	>10 years	2.30	1.00–5.40	0.049
Body condition score	>5 (Reference)	1.00	—	—
	>3–≤5	1.20	0.65–2.20	0.55
	≤3	3.10	1.05–9.20	0.040
Season	Winter (Reference)	1.00	—	—
	Summer	3.10	1.45–6.60	0.003
Ivermectin prophylaxis	Yes (Reference)	1.00	—	—
	No	2.05	1.18–3.55	0.011
Imidocarb prophylaxis	Yes (Reference)	1.00	—	—
	No	1.65	0.88–3.10	0.116
Frequent travelling	No (Reference)	1.00	—	—
	Yes	1.45	0.68–3.10	0.33
Multi-species farm	No (Reference)	1.00	—	—
	Yes	2.35	1.25–4.42	0.008

Note: Aggregated epidemiological data were used to reconstruct analyses to obtain adjusted odds ratios (AORs). Thus, these estimates are tentative approximations and should not be taken as typical regression coefficients with individual variation eliminated or as proof of causal effects.

Mixed seropositivity for *Theileria equi* and *Babesia caballi*

We tested 430 horses for mixed seropositivity with *T. equi* and *B. caballi*; 64 tested positive, for an overall prevalence of 14.88%. Table 6 shows the association between mixed seropositivity with *T. equi* and *B. caballi* and the investigated epidemiological and management-related factors. Horses from various provinces were found to be significantly different based on the type of mixed seropositivity of *T. equi* and *B. caballi* ($\chi^2 = 19.518$, $p = 0.0002$). In Sindh, 24.47% of 94 horses were positive, which was the highest prevalence and was used as the reference. The prevalence in KPK was 21.74% (20/92; 95% CI: 14.54–31.21%), with an OR of 0.86 (95% CI: 0.43–

1.70) compared with Sindh. In Punjab, 20 of 200 horses were positive, corresponding to a prevalence of 10.00% (95% CI: 6.57–14.94%), with significantly lower odds of mixed infection than in Sindh (OR = 0.34; 95% CI: 0.18–0.66). Only 1 of 44 horses (2.27%; 95% CI: 0.40–11.81%) was positive in G-B, and the odds of mixed infections were significantly lower than in Sindh (OR = 0.07; 95% CI: 0.01–0.55). For mixed infection (*T. equi* and *B. bovis*), the highest prevalence was again recorded in D.I. Khan (55.55%), followed by Peshawar, Hyderabad, Sanghar, Mirpur Khas, and Karachi. There was also considerable variation in the geographical distribution of mixed infection, with several districts having no mixed-infected animals. But this district-wise variation was not statistically significant ($\chi^2 = 14.60$, $df = 11$, $p = 0.2017$).

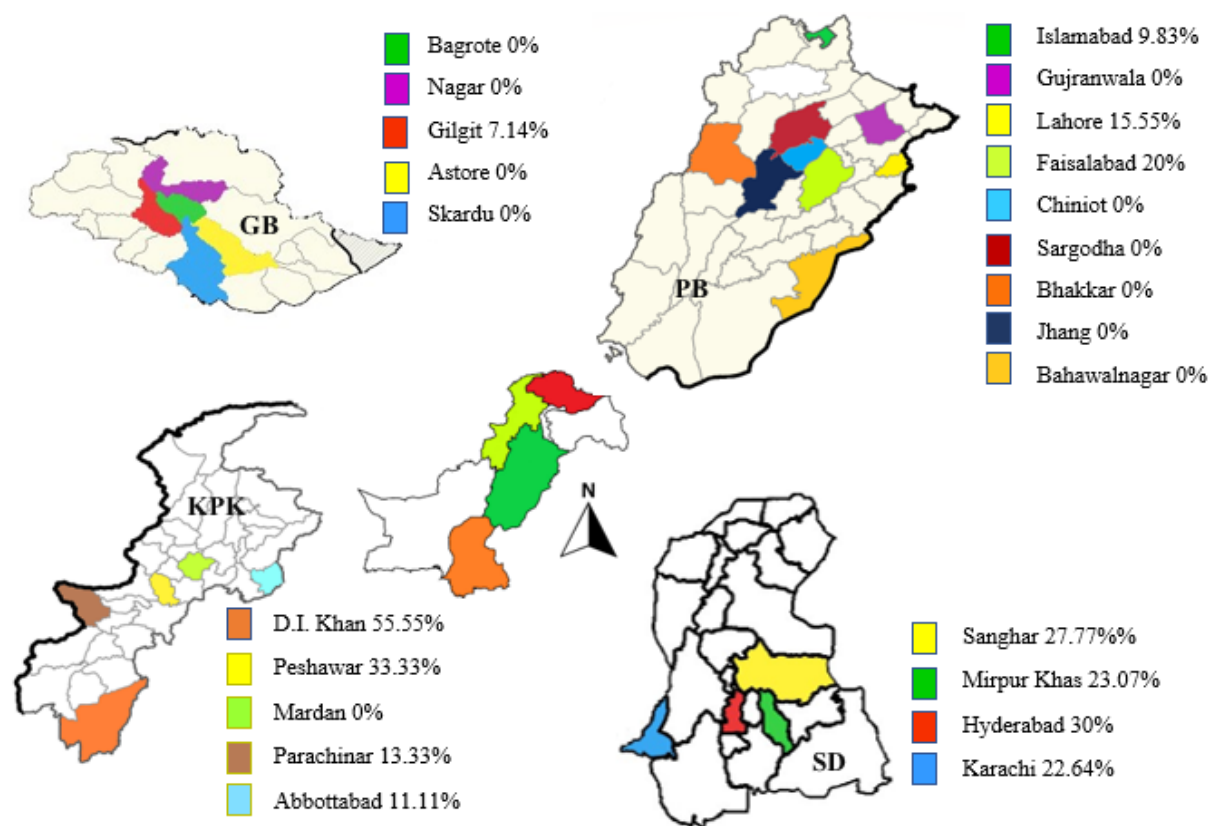


Figure-3. Mixed seropositivity for *Theileria equi* and *B. caballi* in different districts of Pakistan.

There was no statistically significant difference between male and female horses in the prevalence of mixed *T. equi* and *B. caballi* seropositivity ($\chi^2 = 0.231$, $p = 0.6312$). Among males, 36 of 230 horses were positive, giving a prevalence of 15.65% (95% CI: 11.53–20.91%), whereas 28 of 200 females were positive, corresponding to a prevalence of 14.00% (95% CI: 9.87–19.49%). Since males had the higher prevalence, they were considered the reference category. Female horses had an OR of 0.88 (95% CI: 0.51–1.50); no difference in the odds of mixed seropositivity was observed between the sexes. Age group was significantly associated with mixed seropositivity of *T. equi* and *B. caballi* ($\chi^2 = 15.011$, $p = 0.0005$). Horses older than 10 years were the most numerous (35/155) and served as the reference group. Horses aged >5–10 years had a prevalence of 13.37% (25/187; 95% CI: 9.22–18.99%) and significantly lower odds of mixed seropositivity (OR = 0.53; 95% CI: 0.30–0.93). The lowest prevalence was in horses <5 years, with 4 of 88 (4.55%, 95% CI: 1.78–11.11%) being infected, and horses <5 years were less likely to be mixed-infected than horses >10 years (OR = 0.16;

95% CI: 0.06–0.48). The association of mixed seropositivity of *T. equi* and *B. caballi* was not statistically significant ($\chi^2 = 8.477$, $p = 0.2052$), but there was one association that was seen to vary by breed. Sianen (Shiaen) horses had the highest prevalence (9/45; 20.00%, 95% CI: 10.90–33.82%), so they were the reference breed. The prevalence was 18.82% in Indigenous (Desi) horses (32/170; 95% CI: 13.66–25.36%; OR = 0.93; 95% CI: 0.41–2.12), 17.50% in Arab horses (7/40; 95% CI: 8.75–31.95%; OR = 0.85; 95% CI: 0.28–2.54), and 16.67% in Anmol horses (1/6; 95% CI: 3.01–56.35%; OR = 0.80; 95% CI: 0.08–7.73). Suffolk horses showed a prevalence of 12.50% (2/16; 95% CI: 3.50–36.02%; OR = 0.57; 95% CI: 0.11–2.98), while Thoroughbred horses had a prevalence of 8.72% (13/149; 95% CI: 5.17–14.35%; OR = 0.38; 95% CI: 0.15–0.97). None of the 4 Pony horses tested were found to have a positive result. BCS was significantly associated with mixed seropositivity with *T. equi* and *B. caballi* ($\chi^2 = 15.895$, $p = 0.0004$). Horses were classified as having a BCS ≤ 3 had the most severe cases and were chosen as the reference group, as 10 of 25 horses were positive (40.00%; 95%

CI: 23.40–59.26%). Horses with a BCS >3 to ≤ 5 had a prevalence of 14.77% (48/325; 95% CI: 11.32–19.04%) and significantly lower odds of mixed seropositivity (OR = 0.26; 95% CI: 0.11–0.61). Horses with a BCS >5 had the lowest prevalence (7.50%; 6/80; 95% CI: 3.48–15.41%) and significantly reduced odds of mixed seropositivity compared with horses with BCS ≤ 3 (OR = 0.12; 95% CI: 0.04–0.39). The distribution of season and mixed seropositivity of *T. equi* and *B. caballi* was significantly associated ($\chi^2 = 17.919$, $p < 0.0001$). Summer was chosen as the reference category because prevalence was significantly higher, with 59/300 horses positive (19.67%; 95% CI: 15.56–24.54%). During the winter, however, only 5 of the 130 horses were positive, resulting in a prevalence of 3.85% (95% CI: 1.65–8.69%). Horses examined in winter were more than 5 times less likely to be found to be infected with both than were horses examined in summer (OR = 0.16; 95% CI: 0.06–0.42).

Ivermectin use was significantly associated with mixed seropositivity with *T. equi* and *B. bovis* ($\chi^2 = 32.898$, $p < 0.0001$). The highest-prevalence group and reference group were horses that had not received ivermectin (46/170; 27.06% (95% CI: 20.94–34.19%)). A prevalence of 6.92% (95% CI: 4.42–10.68%) was found in 18 horses that tested positive among 260 horses that were treated with ivermectin. Horses treated with ivermectin had significantly lower odds of mixed seropositivity than untreated horses (OR = 0.20; 95% CI: 0.11–0.36). Also, there was a significant association between imidocarb prophylaxis and mixed seropositivity of *T. equi* and *B. caballi* ($\chi^2 = 9.819$, $p = 0.0017$). Horses that received no imidocarb had the highest prevalence (18.62%; 54/290; 95% CI: 14.56–23.50%) and were the reference group. Among horses receiving imidocarb, 10 of 140 were positive, giving a prevalence of 7.14% (95% CI: 3.93–12.65%). The odds of mixed seropositivity were significantly lower among horses receiving imidocarb (OR = 0.34; 95% CI: 0.17–0.68) than among those that did not receive prophylaxis. There was a significant association between acaricide use and mixed seropositivity of *T. equi* and *B. caballi* ($\chi^2 = 32.898$, $p < 0.0001$). Horses that were from premises where acaricides were not used had the highest prevalence, with 46 of 170 horses being positive (27.06%; 95% CI: 20.94–34.19%), and this group was used as the reference. In comparison, 18 of 260 horses from premises where acaricides were used were positive (6.92%; 95% CI: 4.42–10.68%). Horses

on premises where acaricides were used had significantly lower odds of mixed seropositivity than horses on premises where acaricides were not used (OR = 0.20; 95% CI: 0.11–0.36).

Horses that traveled frequently had a higher prevalence of mixed seropositivity with *T. equi* and *B. caballi*; however, this was not statistically significant ($\chi^2 = 2.630$; $P = 0.1049$). Among horses that traveled frequently, 58 of 360 were positive (16.11%; 95% CI: 12.67–20.26%), compared with 6 of 70 among horses that did not travel frequently (8.57%; 95% CI: 3.99–17.47%). The higher prevalence was seen in the frequently traveling group, which was therefore used as the reference category. Horses not involved in frequent travel had an OR of 0.49 (95% CI 0.20–1.18), which showed no statistically significant difference in the odds of seropositivity. No statistical association between the number of horses per herd and the presence of mixed seropositivity of *T. equi* and *B. caballi* was found ($\chi^2 = 5.594$, $p = 0.0610$). The herds with the greatest number of horses (11–50 horses) had the largest prevalence at 38/200 (19.00%; 95% CI: 14.17–25.00%) and were used as the reference. The prevalence among herds containing >50 horses was 12.50% (20/160; 95% CI: 8.24–18.52%), with an OR of 0.61 (95% CI: 0.34–1.10). Herds containing <10 horses had a prevalence of 8.57% (6/70; 95% CI: 3.99–17.47%), with an OR of 0.40 (95% CI: 0.16–0.99) compared with herds containing 11–50 horses. The prevalence of *T. equi* and *B. caballi* mixed seropositivity was statistically similar ($\chi^2 = 4.589$, $p = 0.4681$), but the difference was numerically larger when compared with the purpose of use of horses. The prevalence of horses used for other purposes, such as pets and affiliations, was the highest (8/30; 26.67%; 95% CI: 14.18–44.45%) and was used as a reference category. The prevalence was 17.50% among horses used for ceremony/exhibition (7/40; 95% CI: 8.75–31.95%; OR = 0.58; 95% CI: 0.18–1.84), 16.00% among those used for recreation/dancing (8/50; 95% CI: 8.34–28.51%; OR = 0.52; 95% CI: 0.17–1.59), and 14.62% among polo horses (19/130; 95% CI: 9.56–21.70%; OR = 0.47; 95% CI: 0.18–1.21). Racing/riding horses had a prevalence of 12.50% (15/120; 95% CI: 7.72–19.60%; OR = 0.39; 95% CI: 0.15–1.04), while horses used for tent pegging had the lowest prevalence (11.67%; 7/60; 95% CI: 5.77–22.18%; OR = 0.36; 95% CI: 0.12–1.12).

It was noted that there was a highly significant association between horses kept on a multi-species farm and the mixed seropositivity of *T. equi* and *B.*

caballi ($\chi^2 = 17.243$, $p < 0.0001$). Prevalence was highest on multi-species farms, with 55 of 270 horses testing positive (20.37% [95% CI: 16.00–25.57%]). Conversely, only 9/160 horses on farms with only one species of animal were positive (5.63% [95% CI: 2.99–10.34]). Horses on farms with no other animal species had significantly reduced odds of mixed seropositivity (OR = 0.23; 95% CI: 0.11 – 0.49) compared with horses on multi-species farms. Overall, province, age,

body condition score, season, use of ivermectin, imidocarb prophylaxis, use of acaricides, and multi-species farming were highly significant variables in relation to the prevalence of mixed seropositivity with *T. equi* and *B. caballi*. Sex, breed, frequent traveling, herd size, and the reason for horse ownership were not significantly associated with the presence of mixed seropositivity at the 5% level.

Table-6. Univariable analysis of factors associated with mixed seropositivity of *Theileria equi* and *Babesia caballi* in game horses.

Variable	Category	Positive/Tested	Prevalence (%)	95% CI (%)	OR	95% CI for OR	χ^2	p-value
Province	Sindh (Reference)	23/94	24.47	16.89–34.05	1.00	Reference	19.518	0.0002
	KPK	20/92	21.74	14.54–31.21	0.86	0.43–1.70		
	Punjab	20/200	10.00	6.57–14.94	0.34	0.18–0.66		
	Gilgit Baltistan	1/44	2.27	0.40–11.81	0.07	0.01–0.55		
Sex	Male (Reference)	36/230	15.65	11.53–20.91	1.00	Reference	0.231	0.6312
	Female	28/200	14.00	9.87–19.49	0.88	0.51–1.50		
Age group	>10 year (Reference)	35/155	22.58	16.71–29.78	1.00	Reference	15.011	0.0005
	>5–10 year	25/187	13.37	9.22–18.99	0.53	0.30–0.93		
	<5 year	4/88	4.55	1.78–11.11	0.16	0.06–0.48		
Breed	Siaen (Shiaen) (Reference)	9/45	20.00	10.90–33.82	1.00	Reference	8.477	0.2052
	Indigenous (Desi)	32/170	18.82	13.66–25.36	0.93	0.41–2.12		
	Arab	7/40	17.50	8.75–31.95	0.85	0.28–2.54		
	Anmol	1/6	16.67	3.01–56.35	0.80	0.08–7.73		
	Suffolk	2/16	12.50	3.50–36.02	0.57	0.11–2.98		
	Thoroughbred	13/149	8.72	5.17–14.35	0.38	0.15–0.97		
	Pony	0/4	0.00	0.00–48.99	0.43	0.02–8.64		
Body Condition Score	≤ 3 (Reference)	10/25	40.00	23.40–59.26	1.00	Reference	15.895	0.0004

Variable	Category	Positive/Tested	Prevalence (%)	95% CI (%)	OR	95% CI for OR	χ^2	p-value
	>3 to ≤5 BCS	48/325	14.77	11.32–19.04	0.26	0.11–0.61		
	>5 BCS	6/80	7.50	3.48–15.41	0.12	0.04–0.39		
	Summer (Reference)	59/300	19.67	15.56–24.54	1.00	Reference		
	Winter	5/130	3.85	1.65–8.69	0.16	0.06–0.42		
Ivermectin (tick prophylaxis)	No (Reference)	46/170	27.06	20.94–34.19	1.00	Reference	32.898	<0.0001
	Yes	18/260	6.92	4.42–10.68	0.20	0.11–0.36		
Imidocarb (EP prophylaxis)	No (Reference)	54/290	18.62	14.56–23.50	1.00	Reference	9.819	0.0017
	Yes	10/140	7.14	3.93–12.65	0.34	0.17–0.68		
Use of acaricides	No (Reference)	46/170	27.06	20.94–34.19	1.00	Reference	32.898	<0.0001
	Yes	18/260	6.92	4.42–10.68	0.20	0.11–0.36		
Frequent travelling	Yes (Reference)	58/360	16.11	12.67–20.26	1.00	Reference	2.630	0.1049
	No	6/70	8.57	3.99–17.47	0.49	0.20–1.18		
Heads per herd	11–50 (Reference)	38/200	19.00	14.17–25.00	1.00	Reference	5.594	0.0610
	>50	20/160	12.50	8.24–18.52	0.61	0.34–1.10		
	<10	6/70	8.57	3.99–17.47	0.40	0.16–0.99		
Purpose	Others/Pet/Affiliations (Reference)	8/30	26.67	14.18–44.45	1.00	Reference	4.589	0.4681
	Ceremony/Exhibition	7/40	17.50	8.75–31.95	0.58	0.18–1.84		
	Recreation/Dancing	8/50	16.00	8.34–28.51	0.52	0.17–1.59		
	Polo	19/130	14.62	9.56–21.70	0.47	0.18–1.21		
	Racing/Riding	15/120	12.50	7.72–19.60	0.39	0.15–1.04		
	Tent pegging	7/60	11.67	5.77–22.18	0.36	0.12–1.12		
Multi-species farm	Yes (Reference)	55/270	20.37	16.00–25.57	1.00	Reference	17.243	<0.0001
	No	9/160	5.63	2.99–10.34	0.23	0.11–0.49		

Multivariable analysis of risk factors associated with mixed piroplasm (*T. equi* and *B. caballi*) seropositivity

Multivariable logistic regression was then reconstructed with the following variables: province, age group, body condition score, season, ivermectin prophylaxis, imidocarb prophylaxis, acaricide use and multi-species farming. The overall model was statistically significant (likelihood-ratio $\chi^2 = 369.13$, $df = 11$, $P < 0.001$), suggesting that all the variables incorporated into the model gave a good amount of information regarding the variation in mixed seropositivity positivity. After adjusting for these factors, Province was still highly correlated with piroplasmosis positivity. Horses from Punjab had significantly lower adjusted odds of positivity (AOR = 0.09, 95% CI: 0.01–0.59, $P = 0.012$), that is, there was about a 91% reduction in the odds. Horses from KPK also had lower odds than horses from Sindh (AOR = 0.32) but this was not statistically significant ($P = 0.201$). Likewise, the estimated probability was less than that of Sindh (AOR = 0.21), but the confidence interval was large and contained a value of one ($P = 0.168$) for Gilgit-Baltistan. Therefore, following the adjustment, the highest level of evidence of provincial difference was found for the province of Punjab.

There was a trend for lower odds of seropositivity in younger horses than in horses >10 years of age; however, this association was not statistically significant in the multivariable model. Horses aged >5–10 years had an AOR of 0.43 (95% CI: 0.10–1.95, $P = 0.276$), while horses <5 years had an AOR of 0.20 (95% CI: 0.02–2.20, $P = 0.189$). The point estimates for younger animals appear lower, but the confidence intervals are quite broad after simultaneous adjustment for the other factors, indicating high uncertainty. Body condition score was an independent risk factor for seropositivity. Horses with BCS >3 had substantially lower adjusted odds of piroplasmosis positivity than horses with BCS ≤ 3 (AOR = 0.09, 95% CI: 0.01–0.69, $P = 0.021$). This equates to a 91% drop in the likelihood of being positive. The result suggests that

the reconstructed body condition index was related to the probability of seropositivity. Season was no longer statistically significant after adjustment. Estimated odds were lower for winter sampling compared to summer sampling (AOR = 0.12, 95% CI: 0.01–1.44, $P = 0.095$). However, the point estimate indicated a ~88% decrease in the odds of this association in winter, and the confidence interval overlapped 1, so it did not reach conventional statistical significance. The strongest independent association in the model was with ivermectin prophylaxis. The adjusted odds of a piroplasmosis-positive horse were significantly lower in horses that received ivermectin than in horses that did not receive ivermectin (AOR = 0.06, 95% CI: 0.01–0.37, $P = 0.002$). This equates to a 94% reduction in the risk of positivity. This result confirms that the great difference in the univariate analysis might also be relevant for tick control measures to reduce *B. caballi* exposure. Imidocarb prophylaxis was no longer statistically significant after adjustment. Horses that did not receive prophylaxis were more likely to be positive than horses that received imidocarb (AOR = 2.24, 95% CI: 0.69–6.85, $P = 0.200$); however, the confidence interval was wide and included 1. In the adjusted model, the protective association seen in the univariate analysis was greatly reduced, suggesting other factors contributed to the effect. The use of acaricide was still a significant negative predictor of seropositivity. Horses treated with acaricides had an AOR of 0.11 (95% CI: 0.02–0.55, $P = 0.007$) compared with horses that were not treated with acaricides.

Multi-species farming was not statistically significant after controlling for other factors. There were no differences between horses kept on single-species farms and those kept on multi-species farms in terms of estimated odds (AOR = 0.43, 95% CI: 0.05–3.98, $P = 0.458$). The point estimate suggested a potentially protective association, but the wide confidence interval indicated considerable uncertainty, and the reconstructed model showed no evidence of an independent association.

Table-7. Reconstructed exploratory multivariable analysis of factors associated with mixed piroplasm (*Theileria equi* and *Babesia caballi*) seropositivity.

Variable	Category	Adjusted OR	95% CI	Wald P
Province	Sindh (Reference)	1.00	—	—
	KPK	0.32	0.06–1.83	0.201
	Punjab	0.09	0.01–0.59	0.012
	Gilgit-Baltistan	0.17	0.01–2.13	0.168
Age group	>10 years (Reference)	1.00	—	—
	>5–10 years	0.43	0.10–1.95	0.276
	<5 years	0.20	0.02–2.20	0.189
Body condition score	≤3 (Reference)	1.00	—	—
	>3	0.09	0.01–0.69	0.021
Season	Summer (Reference)	1.00	—	—
	Winter	0.12	0.01–1.44	0.095
Ivermectin prophylaxis	No (Reference)	1.00	—	—
	Yes	0.06	0.01–0.37	0.002
Imidocarb prophylaxis	No (Reference)	1.00	—	—
	Yes	0.41	0.07–2.49	0.330
Acaricide use	No (Reference)	1.00	—	—
	Yes	0.11	0.02–0.55	0.007
Multi-species farm	Yes (Reference)	1.00	—	—
	No	0.43	0.05–3.98	0.458

Note: Aggregated epidemiological data were used to reconstruct analyses to obtain adjusted odds ratios (AORs). Thus, these estimates are tentative approximations and should not be taken as typical regression coefficients with individual variation eliminated or as proof of causal effects.

Discussion

Theileria equi and *Babesia caballi* is one of the two main etiological agents of equine piroplasmosis (EP), an important tick-borne disease of equids. This study provides epidemiological evidence of EP in game horses in Pakistan, a population distinct from the draught and working horses studied in most previous EP studies in Pakistan. The presence of antibodies against *T. equi* in 33.95% of horses, against *B. caballi* in 20.23% of horses, and mixed seropositivity in 14.88% suggest high exposure to both parasites in the investigated population. This relatively high prevalence is particularly important because game horses may be involved in racing, polo, riding, tent pegging, exhibitions, ceremonies, and other activities that involve movement between locations, which could increase the likelihood of exposure to infected ticks and spread infection between equine populations.

The large discrepancies between microscopic and serological detection were an important finding of the present investigation. *T. equi* was identified in 26.51% of horses, and one horse was *B. caballi* and 1 horse was mixed infection by microscopic examination. In indirect ELISA, *T. equi* antibodies were detected in 33.95% of horses, *B. caballi* antibodies in 20.23%, and mixed antibodies in 14.88%. This difference is epidemiologically plausible because microscopic examination relies on detectable numbers of parasites in circulating erythrocytes, while serological tests rely on the host immune response and can therefore detect animals that have been exposed to parasites in the past or have a long asymptomatic period. In addition, microscopic examination may be complicated because of the difficulty in species identification when the level of parasitemia is low or the morphology of the parasites is not very distinguishable. This is similar to reports from endemic equine populations. Aziz and Al-Barwary (2019) identified *T. equi* in only 8.3% of

equids by Giemsa-stained blood smear, and 20.9% by competitive ELISA in Erbil Province, Iraq; *B. caballi* was identified by Giemsa in 1.7% and by competitive ELISA in 11.2% of equids, and mixed infections increased from 0.6% to 6.9% in the same province. Similarly, Baldani et al. (2010) found *T. equi* in only 3.52% of horses by blood-smear microscopy, while 100% were positive by IFAT and 63.53% by nested PCR, with a significant difference between traditional microscopy and the other two methods. This diagnostic discrepancy is further highlighted by recent evidence from South Gujarat, India. Mavadiya et al. (2023) found, by blood-smear examination, that 1.35% of horses were infected with *T. equi*, while 61.69% were seropositive (positive by cELISA testing), and 10% of these were found infected by PCR, which identified the presence of parasite DNA. It is therefore not surprising that the prevalence is considerably higher in the present study than in others where serology is used to measure prevalence in endemic horse populations. The difference is considered to be due to the different biological endpoints between serology and direct detection of the parasite and not contradictory findings. ELISA is positive if antibody is present, and thus it is evidence of previous and/or current exposure to the corresponding parasite; but a positive test does not prove that a person is actually infected with the parasite. Specifically, seropositivity is not evidence of circulating parasites at the time of sampling. Direct detection techniques (such as PCR) combined with clinical and epidemiological data would be necessary to confirm presence of active infection or parasitemia. Therefore, the seroprevalence estimates presented in this study should be regarded as evidence of exposure and/or serological evidence of infection, not as a measure of active parasitemia. Therefore, the higher proportion of antibody-positive horses detected by serology in the present study is consistent with findings from other endemic horse population and should be interpreted in the context of different biological endpoints measured by serological and microscopic methods. This should therefore be interpreted as reflecting different biological endpoints between serology and direct parasite detection, not as contradictory findings. A positive ELISA result is mainly indicative of past or current exposure and immune response. Still, it is not necessarily indicative of active circulation of organisms at the time of sampling. This is especially true for *T. equi* because apparently healthy horses can be infected as carriers,

and parasite loads may not remain constant over time, as shown in a large group of apparently healthy horses tested by Tirosh-Levy et al. (2020) using PCR, qPCR, and serological methods. In a recent multicountry study of horses and donkeys from Greece, Italy and Israel, Jongejan et al. (2024) showed good agreement between a rapid antibody test and cELISA, noting that seropositivity does not necessarily indicate current or active infection. Likewise, Bravo-Barriga et al. (2022) reported high cELISA seroprevalence in horses used for endurance training, many of which were clinically silent, stressing that serological reactivation does not necessarily mean overt clinical disease. The results provide further evidence of the importance of serological surveillance to detect antibody-positive animals or possible carriers, including those animals that do not show clinical signs of disease. Thus, the present seroprevalences should perhaps be considered indicators of exposure or serological evidence of infection, not absolute evidence of active parasitemia. Another important epidemiological note is the predominance of *T. equi* over *B. caballi* in the present study. This is similar to results from several recent molecular studies in Asia. A large-scale study of 1353 horses in Mongolia revealed a high prevalence of *T. equi* DNA, which was found in 78.2% of horses, compared to 4.6% for *B. caballi* (Otgonsuren et al., 2024), which indicates that *T. equi* was far more prevalent than *B. caballi* in that horse population. Molecular surveillance of grazing horses in Kyrgyzstan also identified *T. equi* in 7.40% of the horses, and *B. caballi* was not detected (Altay et al., 2024). The relative importance of the two piroplasms could be quite different from one geographical region to another and from one herd of horses to another. So, the abundance of *T. equi* in Pakistan may reflect differences in vector competence, ecological suitability, persistence, or cumulative exposure, rather than a common trait across all equine populations.

The high prevalence of *T. equi* reported in the present study is also similar to the recent findings of India. Bhojani et al. (2023) conducted an overall seroprevalence study on *T. equi* in horses of Rajasthan by ELISA and reported 49.66% seroprevalence and concluded that *T. equi* is endemic in the region. The study also showed subclinical exposure alongside clinically evident disease, identifying clinically infected horses with anemia and biochemical changes and demonstrating a range of clinical infection. The lower seroprevalence in the current Pakistani population could be attributed to variations in

environmental factors, tick density, management practices, sampling sites, and horse density. These differences are natural, as EP transmission is highly influenced by local ecological conditions.

The present study detected geographical heterogeneity. The seroprevalence of *T. equi* was highest in Punjab (43.0%), followed by Sindh (35.1%) and Khyber Pakhtunkhwa (4.5%), while it was significantly lower in Gilgit-Baltistan (4.5%). This geographical pattern was also different in *B. caballi*, wherein the seroprevalence rate was highest in Sindh (29.8%), followed by Khyber Pakhtunkhwa (26.1%), Punjab (17.0%), and Gilgit-Baltistan (2.3%). The results suggest that the occurrence of the two parasites may be different under local ecological and management factors. This spatial heterogeneity has been also shown in recent research in Central Asia. In Kyrgyzstan, Atabek et al. (2024) reported high diversity of *T. equi* and *B. caballi* in terms of prevalence of different molecules and genotypic composition in horses, indicating geographical structuring of equine piroplasm populations.

The low prevalence in the GB province might relate to the less favorable environment that promotes abundance and activity of competent tick vectors. However, this interpretation is only a hypothesis, as ticks were not collected and characterized in the present study. The spatial distribution of tick-borne pathogens can be influenced by environmental factors like temperature, precipitation, presence of appropriate wildlife or domestic hosts, vegetation cover etc. which can impact abundance and exposure to ticks. A recent French study of draught horses showed significant spatial variability in *B. caballi* and *T. equi* infection, and that temperature, rainfall, vegetation cover, and altitude are important factors associated with tick infestation, with *T. equi* infection showing a stronger association with environmental conditions (Nadal et al., 2022). In Pakistan, significant differences in tick infestation and tick species composition were found between agro-ecological zones and even among equines, underscoring the role of geographical and ecological conditions in tick distribution (Hussain et al., 2023). Therefore, the lower prevalence recorded in this study in Gilgit-Baltistan could be due to less suitability for relevant vector populations, but other factors such as differences in equine management, density of hosts, sampling distribution, or animal movement cannot be ruled out. Future studies in Pakistan should incorporate equine serological and molecular

surveillance and systematically collect, identify, and test ticks for piroplasmosis, alongside environmental and climatic data, to evaluate whether provincial variations in equine piroplasmosis correlate with variations in vector abundance and infection.

Overall, age was significantly correlated with exposure to both parasites. The prevalence of *T. equi* and *B. caballi* was highest in horses over 10 years old (56.8% and 28.4%, respectively), and the lowest in the youngest horses. Mixed infection occurred more frequently in older horses as well. This dose-response relationship is potentially biological, since age is positively correlated with the number of infected ticks. Older horses have more opportunities to be exposed to infected vectors during their lifetime and are therefore more likely to acquire and maintain antibodies. The cumulative effect can be further exacerbated by persistent *T. equi* infection and the ability of recovered horses to remain carriers. The prevalence of *T. equi* has also been higher in horses older than 5 years in recent molecular surveillance in Kyrgyzstan, but this difference was not statistically significant (Altay et al., 2024). This stronger age association in the present study may reflect the longer exposure period in the game-horse population in Pakistan.

Sex was not significantly correlated with seropositivity for either parasite or mixed infection. The high prevalence in both males and females indicates that infection by infected vectors is more affected by environmental and management conditions than sex (Karatepe et al., 2009). Male and female game horses may live in similar housing and have similar grazing/transport/sporting environments. Therefore, significant geographical and seasonal differences that were found in this study are not likely to be due to sex distribution differences.

Season was one of the most significant epidemiological factors in this investigation. Seroprevalence of both parasites was significantly higher in summer than in winter. Prevalence for *T. equi* became 6.9% during winter and 45.7% during summer, and *B. caballi* went up from 6.2% to 26.3% in summer compared to winter. The same was observed in mixed infection, which rose from 3.85% in winter to 19.67% in summer. This may reflect a seasonal pattern and suggests higher transmission in warmer seasons. Temperature and environmental conditions are very important factors affecting the biology of Ixodid ticks and can lead to higher activity levels of these vectors in favorable seasons, resulting in a higher probability of transmission of parasites

(Nadal et al., 2022). Recent studies also showed a strong correlation between tick exposure and *T. equi* seropositivity (Tirosch-Levy et al., 2020), reinforcing the importance of environmental conditions.

The relationship between tick exposure and EP is especially strong in a recent study in southern Brazil. Pereira et al. (2023) compared horses in a *Rhipicephalus microplus*-free area and a tick-infested area, and concluded that *T. equi* seroprevalence was 6% in the *Rhipicephalus microplus*-free herd and 70% in the tick-infested herd. In addition, previous observation of ticks on horses approximately quadrupled the likelihood of *T. equi* seropositivity, and observation of ticks on cattle sharing paddocks with horses approximately tripled the likelihood of *T. equi* seropositivity. The present findings substantiate the above interpretation with a considerable extent of external support, as there is a seasonal pattern, as observed in Pakistan, which could be the result of increased exposure and tick activity at least in part.

Results indicated that use of acaricides was correlated with lower seropositivity for the present study. The observed seroprevalence was lower for *B. caballi*, *T. equi* and mixed seropositivity in horses on premises where acaricides were used than in those on premises where acaricides were not used. In exploratory adjusted analysis of mixed seropositivity, the association was also retained when using acaricide. Biologically these findings are consistent with the tick exposure importance in equine piroplasmosis as shown by previous epidemiological studies. At the present time, however, the results of the observations made do not mean that acaricide treatment per se prevented infection or exposure. Acaricide use might be linked to other preventative and management measures such as veterinary supervision, pasture and housing management, awareness of owner, hygiene practices, routine health checks and other tick management strategies. Thus, applying acaricides might be an indicator of a wider spectrum of preventive-management practice, as opposed to a single cause-effect exposure. Confounding cannot be ruled out because of the potentially correlated practices we were not able to experimentally control and/or comprehensively measure in the present study. To evaluate the independent effect of acaricide use on equine piroplasm exposure, longitudinal or intervention-based studies would be required that contained detailed information on the type of acaricide, use frequency, timing of use, duration of use, and other tick-control measures used. A similar

epidemiological association between the tick-control practices and tick borne Theileria infection has also been reported in Pakistan. The use of acaricide, along with tick infestation, age, herd size and season was found to be associated with tropical theileriosis in Jhang and Bhakkar in cattle and buffaloes respectively by Atif et al. (2025). Though the host and parasite were different from those studied in the current study, the results of these studies indicate the general applicability of tick-control practices as epidemiological factors in Pakistani livestock systems. In a larger serological survey in Southern Spain, tick presence was significantly associated with increased *T. equi* and *B. caballi* seroprevalence in multivariable analyses, and the authors recommended including tick-control programs as part of EP management (García-Bocanegra et al., 2013). A subsequent systematic review and meta-analysis also confirmed that tick exposure is an epidemiologic risk factor for piroplasmosis in horses (Nadal et al., 2022). These results also align with Pereira et al. (2023), who found that horses in tick-exposed environments were significantly more exposed to *T. equi*. However, due to the lack of experimental assignment of acaricide use, the observed association cannot provide direct evidence of a protective effect. Other differences in premises using acaricides include veterinary supervision, pasture management, owner awareness, and animal hygiene and housing. Additionally, acaricides do not necessarily prevent piroplasm infections, and the results should not be regarded as proof that acaricide use alone prevents EP, as with other tick-control strategies (Elsawy et al., 2023; Mendoza et al., 2024).

The same holds for the strong association between ivermectin prophylaxis and low seroprevalence. The prevalence of *T. equi*, *B. caballi*, and mixed infection was significantly lower in horses administered ivermectin. This association was present in exploratory adjusted analyses and was statistically significant, especially for mixed infection. While these results suggest that prophylactic use may reduce exposure, it is important to keep in mind that ivermectin use may reflect broader veterinary management practices and not directly drive the results. Feeding horses may make them susceptible to infection by tick-borne disease-causing organisms, as ticks need to insert their heads into the host's skin and feed on blood to be able to transmit disease-causing organisms. Equine piroplasmosis is tick-borne, and prevention usually relies on good tick control and

broader biosecurity and management (Tamzali, 2013; WOA, 2024). Thus, the documented relationship in this observation study should not be used as proof of a direct prophylaxis effect of ivermectin against *T. equi* or *B. caballi*. Future studies should document the specific drug, dosage, frequency, timing, and duration of tick control, and quantify tick loads and tick-control practices.

The strong association between multi-species farming and EP exposure is another important finding. The prevalence of *T. equi*, *B. caballi*, and mixed infection was significantly higher in horses from multi-species farms compared to horses from single-species farms, especially in univariable analyses. One possible explanation is that multi-species farms may provide environmental and host conditions that promote tick survival and increase horses' exposure to infected vectors. The relevance of this ecological component is further supported by field evidence from south-eastern France, where several species of ixodid ticks infected with *T. equi* and *B. caballi* were found on horses, and tick distribution was influenced by neighboring land-use and environmental factors such as wetlands, pastures, and other landscape features (Rocafort-Ferrer et al., 2022). Likewise, Pereira et al. (2023) found that ticks on cattle in the same paddock were a risk factor for *T. equi* exposure in horses. Thus, multi-species farming could be viewed as a farm-level indicator of increased tick exposure, not necessarily direct transmission from other animal species.

Yet another significant correlation was found between low BCS and seropositivity. Horses with BCS ≤ 3 tended to be more likely to be seropositive for *B. caballi* and mixed seropositive than horses with better BCS, with poor BCS being associated with mixed seropositivity in the exploratory adjusted analysis. This association should be interpreted with caution, and does not necessarily imply that poor body condition predisposes to piroplasm infection. There are a number of explanations. Although not specifically assessed in the current study, poor nutritional status may impact on immune competence and may therefore have been related to increased susceptibility to infection and/or increased persistence of infection. However, a chronic or clinically significant piroplasm infection may lead to loss of body condition due to decreased appetite, anemia, performance or other systemic impacts. Therefore, it is also possible that infection or prior disease causes poor body condition (reverse causation). Moreover, BCS could be the result of other unmeasured

management, nutritional, health or socioeconomic factors that are correlated with both treatment practices and the risk of catching infections. The present study was cross-sectional, and serological positivity does not necessarily indicate present infection, but past infection. In this regard, BCS should be considered as an epidemiological marker for seropositivity, and not as a proven risk factor. Conversely, chronic infection with *T. equi* or *B. caballi* can reduce body condition due to decreased appetite, fat loss, anemia, and depressed performance, as reported in horses chronically infected with equine piroplasm (Wise et al., 2013, Tamzali, 2013, Rothschild, 2013). Because this study was cross-sectional, we could not determine the causal direction of this association. Body condition score may consequently be a host trait, a result of chronic infection, or a sign of other management or health factors. Therefore, BCS is a marker of epidemiological association and not necessarily causal risk.

In the current study, 14.88% of horses were seropositive for both *T. equi* and *B. caballi*, indicating that mixed infection should be carefully considered. Other reports of simultaneous exposure include Hussain et al. (2014), who found antibodies against both parasites in 10.2% of horses sampled in Pakistan, and a study in Brazil, where 50.0% of horses were seropositive for both piroplasms (Vieira et al., 2013). This relatively high mixed seroprevalence may reflect environmental and management conditions that permit transmission of multiple equine piroplasms, particularly in regions where ticks can be competent vectors. Tick infestation has been shown to be a significant risk factor for *T. equi* exposure among equids in Khyber Pakhtunkhwa, Pakistan (Afridi et al., 2017). Higher mixed seropositivity in older horses may partly be due to cumulative exposure over time, which has been associated with *T. equi* seropositivity in other endemic areas (Bartolomé Del Pino et al., 2016; Sigg et al., 2010). This does not imply that mixed seropositivity of the two parasites reflects biological interaction or synergism between them, however, because seropositivity is considered a sign of past exposure. The high mixed seroprevalence in Sindh and Khyber Pakhtunkhwa and the low levels in Gilgit-Baltistan found in the present study may instead be attributed to differences in tick ecology, climatic factors, horse management, and accumulated exposure. Nationwide surveillance in Mongolia also showed extensive co-circulation of *T. equi* and *B. caballi* and high genetic diversity, highlighting the

need to consider multiple piroplasms in endemic areas when evaluating horse populations (Otgonsuren et al., 2024). The seroprevalence and spatial distribution observed suggested high exposure, but alone does not prove that there is transmission maintained or sustained at the population and national level in game horses. The proof would need to include a persistent carrier infection, finding active parasites, the presence of infected competent vectors and the longitudinal dynamics of transmission.

Interestingly, despite numerically higher prevalence of infection in frequently traveling horses, frequency of traveling was not independently associated with infection. Moving horses does not necessarily affect this finding. Equine piroplasmosis is an important tick-borne disease that can be introduced to areas with competent tick vectors by infected or subclinically infected carrier horses (Coulouts et al., 2023; Peckle et al., 2018). However, the epidemiological impact of movement can depend heavily on movement patterns, tick fauna, environmental conditions, and associated management practices. Significant geographical variation in *T. equi* prevalence and relationships with management, pasture access, climate, soil type, age, and other host/environmental factors (Tirosh-Levy et al., 2021; Bartolomé Del Pino et al., 2016). Thus, the current cross-sectional study may reflect interdependence among traveling, age, season, purpose of use, management, and veterinary practices. Movement between regions of differing endemicity and tick ecology would be better substantiated by longitudinal studies of the horses both prior to and after movement.

The results are also relevant to diagnostic surveillance of game horses. Given recent developments in diagnosing equine piroplasmosis, no single diagnostic tool is sufficient to describe equine exposure, infectious status, and carrier status; serological (antibody) and molecular (PCR) results must be considered alongside the epidemiological context. Antibody-based assays are very useful for detecting animals that have been previously exposed and also useful for large-scale surveillance, but do not necessarily mean that the animals are infected with parasites at the time that the antibody test is performed. Molecular assays, on the other hand, provide alternative evidence of parasite DNA and are especially useful in epidemiological investigations where parasitemia is low or subclinical (Wise et al., 2013; Onyiche et al., 2019). Epizootic detection and surveillance can be extended beyond detection by

using PCR-based methods to provide material for sequencing and molecular characterization. Indeed, geographical distribution and spread of the equine piroplasmosis parasites have been highlighted as important by the global analyses, which involved DNA sequencing and genotyping (Tirosh-Levy et al., 2020). PCR and sequencing were also shown to be useful for detecting both *T. equi* and *B. tablonii* and for characterizing geographical variation in their genetic diversity in horse populations in Mongolia and Kyrgyzstan (Otgonsuren et al., 2024; Atabek et al., 2024). Another example is the longitudinal monitoring of *Babesia gibsoni* infection in dogs, where PCR was employed to track the infection over a long period of time (Milošević et al., 2024). This is evidence of a different *Babesia* species and host, but shows how molecular follow-up can be useful for assessing persistent or recurrent haemoparasite infection.

Several potential limitations of the present findings should be acknowledged: First, because of the cross-sectional design, it is not possible to determine temporality and causality. Second, serological positivity may reflect previous or active infection. Thirdly, the lack of PCR means that the number of horses with detectable parasite DNA at the time of sampling cannot be determined. Fourth, tick species were not identified and tick infestation intensity was not measured, so the vector role in transmission cannot be directly assessed. Molecular studies of ticks from livestock are further evidence of the importance of characterizing tick populations that may contain more than one vector-borne pathogen from a single pool of ticks. For instance, Ayan et al. (2024) identified *Anaplasma ovis*, *Ehrlichia canis* and *Ehrlichia chaffeensis* in *Rhipicephalus bursa* ticks from sheep in Turkey. While this pathogen and the geographic context is not a match for the current study, the results highlight the importance of species identification and pathogen screening at the level of the tick and pathogen, respectively, when assessing vector-borne transmission events. Fifth, we used an available-and-proportionate sampling approach rather than probability-based national sampling; therefore, the prevalence estimates may not be nationally representative of all Pakistani game horses. Finally, exploratory adjusted analyses were recreated from aggregated data and should, therefore, be interpreted with caution. There is considerable uncertainty, especially for some adjusted estimates, as reflected by wide confidence intervals.

Further, the identity of the facility was not available for all observations, so that with-in-facility clustering was not formally accounted for and may have led to some non-independence of observations; and also, more than one horse may have been from the same farm or facility, but this information was not available for all observations and so this clustering could not be formally accounted for, resulting in some non-independence of the observations. Treatment-history data were primarily based on farm manager/owner reports and not veterinary treatment records. Therefore, detailed history of the formulation, dosage, frequency, duration, timing and recent use of ivermectin, imidocarb and acaricide was not available. Treatment variables should be interpreted as reported use/nonuse of treatment and not treatment regimens. Treatment histories and confirmation of veterinary treatment should be included in future studies.

Conclusion

This study shows that game horses in the areas studied in Pakistan are exposed to equine piroplasmosis to a significant level, and that geographical, seasonal, body-condition and management practices are significantly associated with this. The relatively high seroprevalence of *T. equi* and *B. caballi* and the mixed seropositivity and differences by season and tick-control practice suggest that pressure of exposure is high in some areas. These findings should not be used as direct evidence of current transmission or maintenance of the parasite, however. No assessment of carrier status, molecular assessment for active parasitemia, vector competence or transmission dynamics was carried out in the present study. So how much game horses contribute to the national level of sustained parasite transmission remains to be seen. The difference in detection by microscopy and serology observed also underlines the need for complementary methods of detection. A comprehensive surveillance approach that includes serology for exposure evaluation, amplification of parasite DNA by PCR, systematic collection and identification of ticks, evaluation of the vector infection and competence, and longitudinal follow-up would provide a better understanding of the dynamics of transmission and maintenance of the parasite in Pakistan. In terms of management, it may be necessary to focus on tick-control practices, especially when there is an increased seasonal risk for exposure to ticks. Further molecular, vector and longitudinal

studies are required to elucidate the role of game horses in the epidemiology and transmission of equine piroplasmosis in Pakistan.

Disclaimer: None.

Conflicts of Interest: None.

Source of Funding: This research received no external funding.

Ethical Approval Statement

Not applicable.

Contribution of Authors

Saqib M, Ashfaq K & Sajid MS: Conceptualization and writing—review and editing.

Saqib M & Tayyab MH: Methodology, validation and investigation.

Tayyab MH: Formal analysis, visualization, data curation and writing—original draft preparation.

Saqib M: Supervision and data interpretation.

All authors read and approved the final draft of the manuscript.

References

- Afridi MJK, Mian AH, Saqib M, Abbas G, Ali J, Mansoor MK, Sial AUR, Rasheed I and Hussain MH, 2017. Seroprevalence and risk factors for *Theileria equi* infection in equines from Khyber Pakhtunkhwa Province, Pakistan. Iran. J. Parasitol. 12(4): 597–605.
- Ahmed Z, Ali A, Waqas M, Ahmed I, Anwar N, Malik MI, Jabbar A, Hussain A and Nawaz M, 2024. Molecular identification and characterization of piroplasms infecting cattle in Azad Kashmir. Pak. Vet. J. 44(3): 611–618. <http://dx.doi.org/10.29261/pakvetj/2024.112>
- Altay K, Erol U, Sahin OF, Ulucesme MC, Aytmirzakizi A and Aktas M, 2024. Survey of tick-borne pathogens in grazing horses in Kyrgyzstan: phylogenetic analysis, genetic diversity, and prevalence of *Theileria equi*. Front. Vet. Sci. 11: 1359974. <https://doi.org/10.3389/fvets.2024.1359974>
- Atabek B, Zhyldyz A, Aitakin K, Rysbek N, Jailobek O, Ahedor B, Mumbi NNM, Ma Y, Otgonsuren D, Perera WPPSI, Guswanto A, Sivakumar T and Yokoyama N, 2024. Molecular prevalence and genotypic diversity of *Theileria equi* and *Babesia caballi* infecting horses in

- Kyrgyzstan. Parasitol. Int. 102: 102915. <https://doi.org/10.1016/j.parint.2024.102915>
- Atif FA, Arif S, Imran M and Arshad M 2025. Molecular epidemiology and detection of mutation in Cytochrome b gene of *Theileria annulata* associated with buparvaquone resistance in bovines from Punjab, Pakistan. Pak. Vet. J. 45(2): 775-782. <http://dx.doi.org/10.29261/pakvetj/2025.195>
- Ayan A, Aslan Çelik B, Çelik ÖY, Oruç Kılınç Ö, Akyıldız G, Yılmaz AB, Sayın İpek DN, Oktay Ayan Ö, Babaoğlu AR, 2024. First detection of *Ehrlichia chaffeensis*, *Ehrlichia canis*, and *Anaplasma ovis* in *Rhipicephalus bursa* ticks collected from sheep, Turkey. Pol. J. Vet. Sci. 27(1): 85-94. doi: 10.24425/pjvs.2024.149338.
- Aziz KJ and Al-Barwary LTO, 2019. Epidemiological study of equine piroplasmosis (*Theileria equi* and *Babesia caballi*) by microscopic examination and competitive-ELISA in Erbil Province, North-Iraq. Iran. J. Parasitol. 14(3): 404-412. <https://doi.org/10.18502/ijpa.v14i3.1479>
- Baldani CD, Nakaghi AC and Machado RZ, 2010. Occurrence of *Theileria equi* in horses raised in the Jaboticabal microregion, São Paulo State, Brazil. Rev. Bras. Parasitol. Vet. 19(4): 228-232. <https://doi.org/10.1590/S1984-29612010000400007>
- Bartolomé Del Pino LE, Nardini R, Veneziano V, Iacoponi F, Cersini A, Autorino GL, Buono F and Scicluna M, 2016. *Babesia caballi* and *Theileria equi* infections in horses in Central-Southern Italy: sero-molecular survey and associated risk factors. Ticks Tick Borne Dis. 7(3): 462-469. <https://doi.org/10.1016/j.ttbdis.2016.01.011>
- Basit A, Ali M, Hussain G, Irfan S, Saqib M, Iftikhar A, Mustafa I, Mukhtar I and Anwar H, 2020. Effect of equine piroplasmosis on hematological and oxidative stress biomarkers in relation to different seasons in District Sargodha, Pakistan. Pak. Vet. J. 40(1): 43-48. <https://doi.org/10.29261/pakvetj/2020.009>
- Bhojani JR, Chahar A, Dewal SV, Mandal DK, Chauhan LS, Pal Y, Kumar R and Kumar S, 2023. Seroprevalence and risk factor associated with endemicity of *Theileria equi* infection in horses in Rajasthan State, India. Indian J. Anim. Res. 57(4): 480-486. <https://doi.org/10.18805/IJAR.B-4325>
- Bravo-Barriga D, Serrano-Aguilera FJ, Barrasa-Rita R, Habela MÁ, Chacón RB, Ezquerro LJ and Martín-Cuervo M, 2022. Effects of competitive ELISA-positive results of piroplasmosis on the performance of endurance horses. Animals 12(5): 637. <https://doi.org/10.3390/ani12050637>
- Cabete A, Xufre Â, Padre L, Bettencourt E, Nunes T and Gomes J, 2025. Occurrence and risk factors of equine piroplasmosis in Portugal: a five-year retrospective study. Vet. Parasitol. 334: 110378. <https://doi.org/10.1016/j.vetpar.2024.110378>
- Coulthous RM, Sutton DGM and Boden LA, 2023. A risk assessment of equine piroplasmosis entry, exposure and consequences in the UK. Equine Vet. J. 55(2): 282-294. <https://doi.org/10.1111/evj.13579>
- Dahiya R, Salar RK, Mandal KD, Kumar R, Tripathi BN, Pal Y and Kumar S, 2018. Risk factor analysis associated with *Theileria equi* infected equines in semi-arid and sub-humid ecological enzootic zones of India. Vet. Parasitol. Reg. Stud. Rep. 12: 17-21. <https://doi.org/10.1016/j.vprsr.2018.01.005>
- Duaso J, Perez-Ecija A, Martínez E, Navarro A, De Las Heras A and Mendoza FJ, 2025. Assessment of common hematologic parameters and novel hematologic ratios for predicting piroplasmosis infection in horses. Animals 15(10): 1485. <https://doi.org/10.3390/ani15101485>
- Elsawy BSM, Nassar AM, Alzan HF, Bhoora RV, Ozubek S, Mahmoud MS, Kandil OM and Mahdy OA, 2021. Rapid detection of equine piroplasms using multiplex PCR and first genetic characterization of *Theileria haneyi* in Egypt. Pathogens 10(11): 1414. <https://doi.org/10.3390/pathogens10111414>
- Elsawy BSM, Mahmoud MS, Suarez CE and Alzan HF, 2023. Impact of equine and camel piroplasmosis in Egypt: how much do we know about the current situation? Pathogens 12(11): 1318. <https://doi.org/10.3390/pathogens12111318>
- García-Bocanegra I, Arenas-Montes A, Hernández E, Adaszek Ł, Carbonero A, Almería S, Jaén-Téllez JA, Gutiérrez-Palomino P and Arenas A, 2013. Seroprevalence and risk factors associated with *Babesia caballi* and *Theileria equi* infection in equids. Vet. J. 195(2): 172-178. <https://doi.org/10.1016/j.tvjl.2012.06.012>
- Ghani U, Ijaz M, Farooqi SH, Ahmed A, Javed MU, Jabir AA, Rasheed H, Batool M and Talha M, 2024. Molecular epidemiology and phylogeny of *Theileria annulata* based on cytochrome b gene in the bovine population of Pakistan. Pak. Vet.

- J. 45(1): 434-439.
<http://dx.doi.org/10.29261/pakvetj/2024.284>
- Hussain MH, Saqib M, Raza F, Muhammad G, Asi MN, Mansoor MK, Saleem M and Jabbar A, 2014. Seroprevalence of *Babesia caballi* and *Theileria equi* in five draught equine populated metropolises of Punjab, Pakistan. Vet. Parasitol. 202(3-4): 248-256.
<https://doi.org/10.1016/j.vetpar.2014.01.026>
- Hussain N, Shabbir RMK, Ahmed H, Afzal MS, Ullah S, Ali A, Irum S, Naqvi SK, Yin J and Cao J, 2023. Prevalence of different tick species on livestock and associated equines and canine from different agro-ecological zones of Pakistan. Front. Vet. Sci. 9: 1089999.
<https://doi.org/10.3389/fvets.2022.1089999>
- Jongejan F, Du C, Papadopoulos E, Blanda V, Di Bella S, Cannella V, Guercio A, Vicari D, Tirosh-Levy S, Steinman A, Baneth G, van Keulen S, Hulsebos I, Berger L and Wang X, 2024. Diagnostic performance of a rapid immunochromatographic test for the simultaneous detection of antibodies to *Theileria equi* and *Babesia caballi* in horses and donkeys. Parasit. Vectors 17(1): 160.
<https://doi.org/10.1186/s13071-024-06253-1>
- Kalantari M, Sharifiyazdi H, Ghaemi M, Ghane M and Nazifi S, 2022. *Theileria equi* in the horses of Iran: molecular detection, genetic diversity, and hematological findings. Vet. Parasitol. Reg. Stud. Rep. 36: 100792.
<https://doi.org/10.1016/j.vprsr.2022.100792>
- Karatepe B, Karatepe M, Cakmak A, Karaer Z and Ergün G, 2009. Investigation of seroprevalence of *Theileria equi* and *Babesia caballi* in horses in Nigde province, Turkey. Trop. Anim. Health Prod. 41(1): 109-113.
<https://doi.org/10.1007/s11250-008-9164-z>
- Mavadiya SV, Patel RM, Mehta SA, Vagh AA, Kalyan IH, Solanki JB, Dangar NS and Patel DR, 2023. Prevalence of *Theileria equi* and *Babesia caballi* infections in horses in South Gujarat, India. Indian J. Anim. Res. 57(10): 1389-1392.
<https://doi.org/10.18805/IJAR.B-4392>
- Mendoza FJ, Pérez-Écija A, Kappmeyer LS, Suarez CE and Bastos RG, 2024. New insights in the diagnosis and treatment of equine piroplasmosis: pitfalls, idiosyncrasies, and myths. Front. Vet. Sci. 11: 1459989.
<https://doi.org/10.3389/fvets.2024.1459989>
- Milošević S, Božović Ilić A, Magaš V, Sukara R, Tomanović S, Radaković M, Spariosu K, Kovačević Filipović M and Francuski Andrić J, 2024. First clinical evidence with one-year monitoring of *Babesia gibsoni* mono-infection in two dogs from Serbia. Pak. Vet. J. 44(4): 1315-1321.
<http://dx.doi.org/10.29261/pakvetj/2024.286>
- Nadal C, Bonnet SI and Marsot M, 2022. Eco-epidemiology of equine piroplasmosis and its associated tick vectors in Europe: a systematic literature review and a meta-analysis of prevalence. Transbound. Emerg. Dis. 69(5): 2474-2498.
<https://doi.org/10.1111/tbed.14261>
- Onyiche TE, Sukanuma K, Igarashi I, Yokoyama N, Xuan X and Thekisoe O, 2019. A review on equine piroplasmosis: epidemiology, vector ecology, risk factors, host immunity, diagnosis and control. Int. J. Environ. Res. Public Health 16(10): 1736.
<https://doi.org/10.3390/ijerph16101736>
- Onyiche TE, Taiwo MO, Molefe NI, Biu AA, Luka J, Omeh IJ, Yokoyama N and Thekisoe O, 2020. Equine piroplasmosis: an insight into global exposure of equids from 1990 to 2019 by systematic review and meta-analysis. Parasitology 147(13): 1411-1424.
<https://doi.org/10.1017/S0031182020001407>
- Otgonsuren D, Amgalanbaatar T, Narantsatsral N, Enkhtaivan B, Munkhgerel D, Zoljargal M, Davkharbayar B, Myagmarsuren P, Battur B, Battsetseg B, Sivakumar T and Yokoyama N, 2024. Epidemiology and genetic diversity of *Theileria equi* and *Babesia caballi* in Mongolian horses. Infect. Genet. Evol. 119: 105571.
<https://doi.org/10.1016/j.meegid.2024.105571>
- Peckle M, Pires MS, Silva CBD, Costa RLD, Vitari GLV, Senra MVX, Dias RJP, Santos HA and Massard CL, 2018. Molecular characterization of *Theileria equi* in horses from the state of Rio de Janeiro, Brazil. Ticks Tick Borne Dis. 9(2): 349-353.
<https://doi.org/10.1016/j.ttbdis.2017.11.011>
- Pereira MR, Trein CR, Webster A, Dallagnol B, Gonchoroski GZ, Pellegrini DP, Doyle R, Klafke G and Reck J, 2023. Comparison of seroprevalence and identification of risk factors for *Theileria equi* in horses from vector-free and infested areas in southern Brazil. J. Equine Vet. Sci. 126: 104241.
<https://doi.org/10.1016/j.jevs.2023.104241>
- Rocafort-Ferrer G, Leblond A, Joulie A, René-Martellet M, Sandoz A, Poux V, Pradier S, Barry S, Vial L and Legrand L, 2022. Molecular assessment of *Theileria equi* and *Babesia*

- caballi* prevalence in horses and ticks on horses in southeastern France. *Parasitol. Res.* 121(3): 999–1008. <https://doi.org/10.1007/s00436-022-07441-7>
- Rothschild CM, 2013. Equine piroplasmosis. *J. Equine Vet. Sci.* 33(7): 497–508. <https://doi.org/10.1016/j.jevs.2013.03.189>
- Scoles GA and Ueti MW, 2015. Vector ecology of equine piroplasmosis. *Annu. Rev. Entomol.* 60: 561–580. <https://doi.org/10.1146/annurev-ento-010814-021110>
- Sigg L, Gerber V, Gottstein B, Doherr MG and Frey CF, 2010. Seroprevalence of *Babesia caballi* and *Theileria equi* in the Swiss horse population. *Parasitol. Int.* 59(3): 313–317. <https://doi.org/10.1016/j.parint.2010.02.005>
- Sumbria D, Das Singla L and Sharma A, 2016. *Theileria equi* and *Babesia caballi* infection of equids in Punjab, India: a serological and molecular survey. *Trop. Anim. Health Prod.* 48(1): 45–52. <https://doi.org/10.1007/s11250-015-0917-1>
- Tamzali Y, 2013. Equine piroplasmosis: an updated review. *Equine Vet. Educ.* 25: 590–598. <https://doi.org/10.1111/eve.12070>
- Thabet NF, Kandil OM and Fararh KM, 2024. Impact of *Theileria equi* infection on Arabian stallion fertility: Serological and hormonal perspectives. *Int. J. Vet. Sci.* 13(6): 1002–1008. <https://doi.org/10.47278/journal.ijvs/2024.196>
- Tirosh-Levy S, Gottlieb Y, Mazuz ML, Savitsky I and Steinman A, 2020. Infection dynamics of *Theileria equi* in carrier horses is associated with management and tick exposure. *Ticks Tick Borne Dis.* 11(6): 101508. <https://doi.org/10.1016/j.ttbdis.2020.101508>
- Tirosh-Levy S, Mazuz ML, Savitsky I, Pinkas D, Gottlieb Y and Steinman A, 2021. Serological and molecular prevalence of *Babesia caballi* in apparently healthy horses in Israel. *Pathogens* 10(4): 445. <https://doi.org/10.3390/pathogens10040445>
- Vieira TS, Vieira RF, Finger MA, Nascimento DA, Sicupira PM, Dutra LH, Deconto I, Barros-Filho IR, Dornbusch PT, Biondo AW and Vidotto O, 2013. Seroepidemiological survey of *Theileria equi* and *Babesia caballi* in horses from a rural and from urban areas of Paraná State, southern Brazil. *Ticks Tick Borne Dis.* 4(6): 537–541. <https://doi.org/10.1016/j.ttbdis.2013.07.005>
- Wang M, Guo W, Igarashi I, Xuan X, Wang X, Xiang W and Jia H, 2014. Epidemiological investigation of equine piroplasmosis in China by enzyme-linked immunosorbent assays. *J. Vet. Med. Sci.* 76(4): 549–552. <https://doi.org/10.1292/jvms.13-0477>
- Wise LN, Kappmeyer LS, Mealey RH and Knowles DP, 2013. Review of equine piroplasmosis. *J. Vet. Intern. Med.* 27(6): 1334–1346. <https://doi.org/10.1111/jvim.12168>
- Wise LN, Pelzel-McCluskey AM, Mealey RH and Knowles DP, 2014. Equine piroplasmosis. *Vet. Clin. North Am. Equine Pract.* 30(3): 677–693. <https://doi.org/10.1016/j.cveq.2014.08.008>
- World Organisation for Animal Health (WOAH), 2024. Terrestrial Animal Health Code. Chapter 12.7. Infection with *Theileria equi* and *Babesia caballi* (equine piroplasmosis). World Organisation for Animal Health, Paris, France.
- World Organisation for Animal Health (WOAH), 2025. International competition horse movement. World Organisation for Animal Health, Paris, France.
- Zhao S, Wang H, Zhang S, Xie S, Li H, Zhang X and Jia L, 2020. First report of genetic diversity and risk factor analysis of equine piroplasm infection in equids in Jilin, China. *Parasit. Vectors* 13(1): 459. <https://doi.org/10.1186/s13071-020-04338-1>