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Original Article

Assessment of *Babesia* spp. prevalence in various domestic animals across Southern Punjab, Pakistan

Avaliação de *Babesia* spp. prevalência em vários animais domésticos no sul de Punjab, Paquistão

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Abstract

Parasitic diseases, notably babesiosis, exert a substantial impact on the global cattle industry, posing challenges to commerce, economies, and human health. This study, conducted in Southern Punjab, Pakistan, aimed to assess the prevalence of *Babesia* spp. across various livestock species using microscopic and PCR methods. A total of 180 blood samples (60 from each district) were systematically collected from apparently healthy animals, with 36 samples obtained from each domestic animal species, including camel, cattle, buffalo, goat, and sheep, noting that 12 samples were collected from each district for each animal species. Overall prevalence was determined to be 32.8% (59/180), with varying rates among species: 25.0% in cattle, 41.66% in buffalo, 30.55% in goats, 33.3% in sheep, and 33.3% in camels. Microscopic examination revealed slightly varied infection rates among large and small domestic animals (22.2%), while PCR results indicated a 32.8% overall infection rate in both large and small domestic animals, with no statistical significance. District-wise analysis showed regional variations, with Muzaffargarh recording a prevalence rate of 23.33% through microscopic examination, while Lodhran and Bahawalpur recorded 21.67%. PCR results revealed higher rates (38.33%, 26.67%, and 33.33%, respectively), underlining the importance of employing PCR for accurate detection. Examining ruminant types, large ruminants exhibited a 32.4% infection rate, while small domestic animals showed 33.3%, with no significant difference (p=0.897). District-wise prevalence showcased significant variation, with Muzaffargarh demonstrating a 25% prevalence, Lodhran 22%, and Bahawalpur 22%, through microscopic examination. PCR results displayed 38.33%, 27%, and 33.3%, respectively, with no statistical significance. Detailed analysis of individual districts highlighted variations in infection rates among camels, cattle, buffalo, goats, and sheep. The binomial test indicated significant differences through microscopic analysis (P=0.011) but non-significant variations through PCR (P=0.065), emphasizing the precision of PCR. Regional variations in prevalence, notably with Punjab exhibiting the highest frequency (33.87%) and KPK the lowest (13.24%), suggest potential influences from varying veterinary practices and environmental factors. This study underscores the pivotal role of PCR alongside microscopy for accurate babesiosis diagnosis. These findings contribute to the broader understanding of babesiosis prevalence, emphasizing the necessity of advanced molecular techniques for informed control measures.

Keywords: *Babesia* spp., domestic animals, microscopy examination, molecular detection, Pakistan.

Resumo

Doenças parasitárias, especialmente a babesiose, exercem um impacto substancial na indústria global de bovinos, apresentando desafios ao comércio, às economias e à saúde humana. Este estudo, realizado no Sul do Punjab, Paquistão, teve como objetivo avaliar a prevalência de *Babesia* spp. em várias espécies de animais domésticos,

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utilizando métodos microscópicos e de PCR. Um total de 180 amostras de sangue (60 de cada distrito) foi coletado sistematicamente de animais aparentemente saudáveis, com 36 amostras obtidas de cada espécie de animal doméstico, incluindo camelo, gado, búfalo, cabra e ovelha, observando que 12 amostras foram coletadas de cada distrito para cada espécie de animal. A prevalência global foi determinada em 32,8% (59/180), com taxas variáveis entre as espécies: 25,0% em bovinos, 41,66% em búfalos, 30,55% em cabras, 33,3% em ovelhas e 33,3% em camelos. A análise microscópica revelou taxas de infecção ligeiramente variadas entre animais domésticos grandes e pequenos (22,2%), enquanto os resultados da PCR indicaram uma taxa global de infecção de 32,8% em ambos os grupos, sem significância estatística. A análise por distrito mostrou variações regionais, com Muzaffargarh registrando uma taxa de prevalência de 23,33% através de exame microscópico, enquanto Lodhran e Bahawalpur registraram 21,67%. Resultados da PCR revelaram taxas mais altas (38,33%, 26,67% e 33,33%, respectivamente), destacando a importância da PCR para detecção precisa. Ao examinar tipos de ruminantes, ruminantes grandes apresentaram uma taxa de infecção de 32,4%, enquanto animais domésticos pequenos mostraram 33,3%, sem diferença significativa (p=0,897). A prevalência por distrito mostrou variação significativa, com Muzaffargarh demonstrando uma prevalência de 25%, Lodhran 22% e Bahawalpur 22%, através de exame microscópico. Resultados da PCR exibiram 38,33%, 27% e 33,3%, respectivamente, sem significância estatística. Análise detalhada dos distritos individuais destacou variações nas taxas de infecção entre camelos, bovinos, búfalos, cabras e ovelhas. O teste binomial indicou diferenças significativas através da análise microscópica (P=0,011), mas variações não significativas através da PCR (P=0,065), enfatizando a precisão da PCR. Variações regionais na prevalência, notadamente com Punjab exibindo a maior frequência (33,87%) e KPK a menor (13,24%), sugerem influências potenciais de práticas veterinárias variadas e fatores ambientais. Este estudo destaca o papel fundamental da PCR juntamente com a microscopia para um diagnóstico preciso da babesiose. Essas descobertas contribuem para uma compreensão mais ampla da prevalência da babesiose, enfatizando a necessidade de técnicas moleculares avançadas para medidas de controle informadas.

Palavras-chave: *Babesia* spp., animais domésticos, exame microscópico, detecção molecular, Paquistão.

1. Introduction

Parasitic infections pose significant challenges to animal production worldwide, causing substantial economic losses and compromising animal welfare (Jamil et al., 2023). These infections encompass a diverse range of pathogens, including protozoa, helminths, and ectoparasites, that can invade various host species, including livestock, companion animals, and wildlife (Attia and Khalifa, 2023). Among these parasites, ticks stand out as particularly problematic ectoparasites that infest a wide array of animals, including reptiles, amphibians, birds, and mammals (Jamil et al., 2023). Their obligatory blood-feeding behavior makes them capable of transmitting a variety of pathogens that can cause severe diseases, such as babesiosis, anaplasmosis, and theileriosis (Kenaw et al., 2023).

Piroplasmosis, a tick-borne disease caused by parasitic protozoa of the genus *Babesia*, poses a significant threat to livestock production in Pakistan. Ticks of the species *Haemophysalis* and *Hyalomma* spp. act as the primary vectors of piroplasmosis, transmitting the parasite between animals (Rehman et al., 2019). This disease has a devastating impact on both small and large domestic animals, causing substantial morbidity and mortality (Ingle, 2019).

In cattle, piroplasmosis is responsible for an estimated 30% fatality rate, making it a major cause of economic losses in the livestock sector (Azam and Shafique, 2017). The high mortality rate among sheep, ranging from 70% to 80%, further underscores the severity of this disease (Mushtaq et al., 2021). The lack of adequate healthcare facilities in many rural areas of Pakistan exacerbates the problem, as affected animals often do not receive timely and effective treatment.

The livestock sector plays a pivotal role in the socio-economic development of Pakistan, contributing significantly to the country's economy and supporting the livelihoods of millions of rural communities (Rehman et al., 2017). According to (Azam and Shafique, 2017), the livestock industry generates around 61.6% of the total value added to agriculture and 16.7% of Pakistan's gross domestic product (GDP). This substantial contribution stems from the production of meat, milk, wool, and other livestock products that are essential for domestic consumption and export.

The World Bank estimates that approximately 40-45 million rural Pakistanis are dependent on livestock for their livelihoods. These individuals often own a small number of animals, typically 2-4 cattle/buffaloes and 6-8 sheep/goats, which provide them with a source of income and food security. It is estimated that 30-40% of their income comes directly from their livestock. The Government of Pakistan (Aslam et al., 2023) recognizes the importance of the livestock sector in driving economic growth in rural areas. The ownership patterns of livestock in rural households underscore the sector's potential to contribute to the economic development of these communities. Sheep and goat husbandry holds particular significance in the rural economy, particularly for non-agricultural households in rural zones (Shahzad et al., 2013). These animals are relatively low-maintenance and can thrive in harsh environments, making them well-suited for pastoralist communities. one researcher have found that sheep and goat rearing is an important source of income and sustenance for these communities (Derar et al., 2023).

The management and control of ticks and tick-borne parasites in Pakistan is of paramount importance, particularly in the province of Punjab, which has the highest population density of any region in the country (Farooqi et al., 2017b). This is because these parasites pose a significant threat to animal health and productivity, and the high population density in Punjab increases the risk of transmission of these diseases (Hussain et al., 2021). The Government of Pakistan (GoP) has implemented several initiatives to address the issue of tick-borne diseases in livestock. These initiatives include education and awareness campaigns, the distribution

of acaricides, and the promotion of tick-resistant breeds. Despite these efforts, the prevalence of tick-borne diseases in Pakistan remains a significant challenge. To effectively control tick-borne diseases, a comprehensive approach is needed that combines preventive measures, such as regular acaricide treatments, with surveillance and monitoring to track the distribution and prevalence of these diseases. The scarcity of research on the prevalence and distribution of tick-borne infections in cattle in Pakistan is a significant impediment to controlling these diseases. Different researchers have all highlighted the need for more comprehensive studies in this area (Khan et al., 2017; Hussain et al., 2022; Zaman et al., 2022).

Clinical signs of babesiosis, a common tick-borne disease in cattle, typically appear 2-3 weeks after tick infestation in an infected animal. However, direct injection of contaminated blood can shorten the incubation period. For instance, the incubation period for *B. bigemina* is 4-6 days, while that for *B. bovis* is 10-12 days (Schnittger et al., 2022). The severity of clinical symptoms can vary depending on the animal's age, with older animals being more susceptible due to their weaker immune systems. *B. bovis* is significantly more virulent than *B. bigemina* and *B. divergens*. Different researchers have reported that *B. bovis* is approximately 100 times more virulent than its counterparts (Gallego-Lopez et al., 2019; Hayati et al., 2020; Surjowardojo et al., 2023).

Investigations into the prevalence of tick-borne infections in various livestock species, including sheep, buffalo, cattle, camels, and goats, are limited in Pakistan (Iseki et al., 2010; Khan et al., 2022).To address these gaps in knowledge, this study aimed to determine the prevalence of *Babesia* spp. in several domestic animal species in three districts (Muzaffargarh, Lodhran, and Bahawalpur) of Southern Punjab, Pakistan, using a combination of microscopic examination and molecular detection using PCR assay.

2. Materials and Methods

2.1. Study area and blood collection

The study was conducted across three diverse districts in South Punjab, Pakistan: Muzaffargarh, Lodhran, and Bahawalpur. To ensure comprehensive representation, a total of 180 blood samples (60 from each district) were systematically collected, with 36 samples obtained from each domestic animal species, including camel, cattle, buffalo, goat, and sheep, noting that 12 samples were collected from each district for each animal species. The sampling period spanned from March to July 2021. Sampling criteria were meticulously designed, considering geographical diversity, accessibility, and livestock populations in the region. The study area is characterized by a dry tropical climate, adding ecological relevance to our investigation. Blood samples were collected using a standardized procedure, where whole blood was drawn from the jugular vein into EDTA-containing vacutainer tubes. To maintain sample integrity during transportation, all samples were promptly placed on ice and transported to the laboratory for further analysis.

2.2. Blood smear detection of Babesia spp.

Babesia species were identified microscopically using the protocol described by (Huber et al., 2017). Fresh blood smears were prepared by placing a blood drop on a clean glass slide and spreading it with a second slide at a 45-degree angle. The smears were fixed in 100% ethanol and stained with Giemsa stain. Slides were examined under a high-magnification oil immersion lens (Nikon, USA) at 100x. Following parasitological assessment, samples were stored at -20°C for PCR analysis.

2.3. DNA extraction and amplification

Genomic DNA was extracted from randomly selected samples using the Wiz prep® Genomic DNA purification kit. A set of oligonucleotide primers, GAU9 (5'-CTGTCGTACCGTTGGTTGAC-3') and GAU10 (Reverse) (3'-CGCACGGACGGAGACCGA-5'), targeting a 541 bp amplicon of the *Babesia* 18S rRNA gene, was used as described by (Larrán et al., 2022). The PCR reaction mixture (25 µl) contained 1X Taq Buffer, 0.2 mM dNTPs, 1.5 mM MgCl2, 2.5 U/l Taq polymerase (Fermentas, UK), 4 mM of each primer, 2 µl of DNA template, and DNase-free deionized water. Blood samples from ruminants with clinical signs of Babesiosis were used as positive controls for DNA extraction. Water served as the negative control, while DNA extracted from a clinically and microscopically positive sample was used as the positive control for PCR. DNA amplification was performed using a thermal cycler (Gene Amp® PCR system 2700 Applied Biosystems Inc., UK). The thermo-profile described by (Larrán et al., 2022) was modified for the current study, with an initial denaturation at 94°C for 10 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 1 minute, and elongation at 72°C for 1 minute. The final extension was done at 72°C for 5 minutes. PCR products were stored at 4°C until further analysis. Electrophoresis was performed on a 2.5% agarose gel followed by visualization under UV light using a Trans illuminator (Bio-Rad, USA).

2.4. Statistical analysis

Data were analyzed using descriptive statistics to determine the prevalence of *Babesia* infection across the study area. This was calculated by dividing the number of infected animals by the total number of animals examined and expressed as percentages. The Chi-Square and Binomial tests were employed to evaluate the strength of the association between variables at 95% confidence intervals for categorical data (Zar, 1999). Data analysis was performed using SPSS statistical software version 20.0. A P-value less than 0.05 was considered statistically significant, while a P-value less than 0.01 was deemed highly significant.

3. Results

3.1. Overall prevalence of Babesia spp. in different animal species

For microscopic identification, 36 blood samples of each animal species were collected from three different districts.

Blood smears were examined under the microscope for the presence of *Babesia* spp. (Figure 1).

The overall prevalence of *Babesia* infection, recorded through PCR assay (Figure 2), was 32.8% (59/180), with varying percentages among animal species: 25.0% (9/36) in cattle, 41.66% (15/36) in buffalo, 30.55% (11/36) in goats, and 33.3% (12/36) in sheep. Camels also showed a prevalence of 33.3% (12/36) (Tables 1 and 2).

3.2. Microscopic and PCR analysis according to domestic animal categories

The microscopic examination revealed that the infection rates differed slightly among various categories of domestic animals. In large domestic animals, the infection rate was 21.3% (23/108), while in small domestic animals, it was slightly higher at 23.6% (17/72). The combined infection rate for both large and small domestic animals was 22.2% (40/180). However, statistical analysis indicated that these variations were not significant (p=0.714). PCR results further delved into the specifics of ruminant types within domestic animals. Large ruminants exhibited an infection rate of 32.4% (35/108), whereas small domestic animals showed a slightly lower rate of 33.3% (24/72). The overall infection rate for both large and small domestic animals, as revealed by PCR, was 32.8% (59/180). Despite these variations, statistical analysis indicated non-significance (p=0.897) in the infection rates among different types of ruminants (Tables 1 and 2).

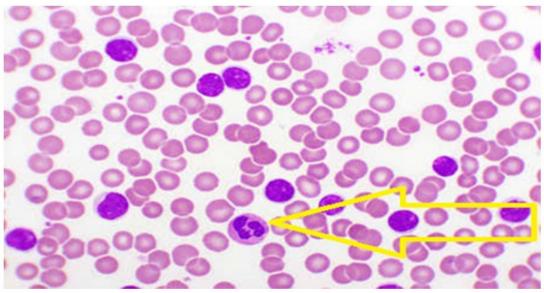

Figure 1. *Babesia* spp. (arrow) in a field stained blood smear from the domestic animals. Magnification 100X.

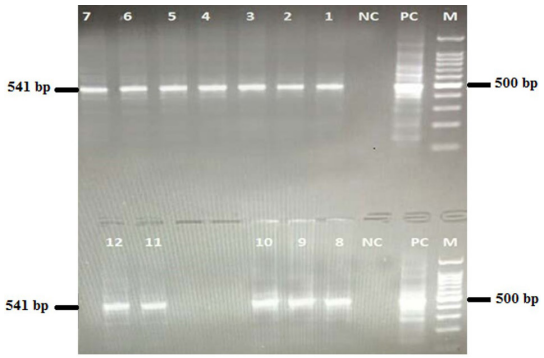

Figure 2. PCR amplification of *Babesia* spp. 18S rRNA partial sequence by using GAU9 and GAU10 primers. Notes: Lane M: 100 bp DNA ladder; Lane 1 to 12: Samples positive for *Babesia* spp.; : Lane M : Marker, Lane PC: Positive control; Lane NC: Negative control.

3.3. Prevalence of Babesia spp. in different districts

Microscopic examination in Muzaffargarh district revealed infection rates of 8.3% (1/12) in camels, 25% (3/12) in cattle, 25% (3/12) in buffalo, 25% (3/9) in goats, and 41.66% (5/12) in sheep. The overall infection rate across all ruminants was 25% (15/60), demonstrating statistical significance (P=0.006). PCR results showed varying rates: 17% (2/12) in camels, 50% (6/12) in cattle, 42% (5/12) in buffalo, 42% (5/12) in goats, and 42% (5/12) in sheep. The total infection rate was 38.33% (6/60), with no statistical significance (P=1.00). In Lodhran district, microscopic analysis indicated infection rates of 25% (3/12) in camels, 33.3% (4/12) in cattle, 8.1% (1/12) in buffalo, 17% (2/12) in goats, and 25% (3/12) in sheep. The overall infection rate was 22% (13/60), showing no statistical significance (P=0.388). PCR results showed infection rates of 25% (3/12) in camels, 42% (5/12) in cattle, 17% (2/12) in buffalo, 25% (3/12) in goats, and 25% (3/12) in sheep. The total infection rate was 27% (16/60), with statistical non-significance (P=0.039). In Bahawalpur, microscopic examination revealed infection rates of 17% (2/12) in camels, 25% (3/12) in cattle, 25% (3/12) in buffalo, 17% (2/12) in goats, and 25% (3/12) in sheep. The overall infection rate was 22% (13/60), showing no statistical significance (P=0.146). PCR results demonstrated infection rates of 33.33% (4/12) in camels, 33.3% (4/12) in cattle, 33.3% (4/12) in buffalo, 33.3% (4/12) in goats, and 33.3% (4/12) in sheep. The total infection rate was 33.3% (20/60), with no statistical significance (P=0.388) (Tables 1 and 2).

3.4. Babesia spp. prevalence in different districts and ruminant types

For camels, microscopic examination reported a total infection rate of 8.3% (1/12) in Muzaffargarh, 25% (3/12) in Lodhran, and 16.66% (2/12) in Bahawalpur. The overall prevalence across all districts was 16.66% (6/36), statistically non-significant (p=1.436). Similarly, PCR results showed a total infection rate of 16.66% (2/12) in Muzaffargarh, 25% (3/12) in Lodhran, and 33.33% (4/12) in Bahawalpur. The overall prevalence across all districts was 25% (9/36), also statistically non-significant (P=0.146). For cattle, microscopic examination indicated a total infection rate of 25% (3/12) in Muzaffargarh, 33.33% (4/12) in Lodhran, and 25% (3/12) in Bahawalpur for cattle. The overall prevalence across all districts was 27.77% (10/36), statistically non-significant (P=0.33). PCR results revealed a total infection rate of 50% (6/12) in Muzaffargarh, 41.66% (5/12) in Lodhran, and 33.33% (4/12) in Bahawalpur. The overall prevalence across all districts was 41.66% (15/36), also statistically non-significant (P=0.388). For buffalo, microscopic examination reported a total infection rate of 25% (3/12) in Muzaffargarh, 8.1% (1/12) in Lodhran, and 25% (3/12) in Bahawalpur. The overall prevalence across all districts was 19.44% (7/36), statistically non-significant (P=0.146). PCR results revealed a total infection rate of 41.66% (5/12) in Muzaffargarh, 16.66% (2/12) in Lodhran, and 33.3% (4/12) in Bahawalpur. The overall prevalence across all districts was 30.5% (11/36), also statistically non-significant (P=0.774). For goats, microscopic examination reported a total infection rate of 25% (3/12) in Muzaffargarh, 16.66% (2/12)

Table 1. Binomial test showing the prevalence in tested large ruminants and methods used for comparison in studied districts.

Animal	Method	Muzaffargarh				Lodhran			Bahawalpur			Total
		Category	Number	Rate (%)	P-value	Number	Rate (%)	P-value	Number	Rate (%)	P-value	
Camel	Microscopic	Positive	1	8	0.006^{S}	3	25	0.146^{NS}	2	17	0.039^{S}	6
	examination	Negative	11	92		9	75		10	83		30
		Total	12	100		12	100		12	100		36
	PCR assay	Positive	2	17	0.039 ^{S}	3	25	0.146 ^{NS}	4	33	0.388^{NS}	9
		Negative	10	83		9	75		8	67		27
		Total	12	100		12	100		12	100		36
Cattle	Microscopic	Positive	3	25	0.146 ^{NS}	4	33	0.388 ^{NS}	3	25	0.146 ^{NS}	10
	examination	Negative	9	75		8	67		9	75		26
		Total	12	00		12	100		12	100		36
	PCR assay	Positive	6	50	1.000^{NS}	5	42	0.774 ^{NS}	4	33	0.388^{NS}	15
		Negative	6	50		7	58		8	67		21
		Total	12	100		12	100		12	100		36
Buffalo	Microscopic	Positive	3	25	0.146 ^{NS}	1	8	0.006 ^{S}	3	25	0.146^{NS}	7
	examination	Negative	9	75		11	92		9	75		19
		Total	12	100		12	100		12	100		36
	PCR assay	Positive	5	42	0.774 ^{NS}	2	17	0.039 ^{S}	4	33	0.388^{NS}	11
		Negative	7	58		10	83		8	67		25
		Total	12	100		12	100		12	100		36

Abbreviations: NS = Non-significant (P > 0.05); S = Significant (P ≤ 0.05); PCR: Polymerase Chain Reaction.

Table 2. Binomial test showing the prevalence in tested small ruminants and methods used for comparison in studied districts.

Animal	Method	Muzaffargarh				Lodhran			Bahawalpur			Total
		Category	Number	Rate (%)	P-value	Number	Rate (%)	P-value	Number	Rate (%)	P-value	
Goat	Microscopic	Positive	3	25	0.146 ^{NS}	2	17	0.039^{S}	2	17	0.039 ^{S}	7
	examination	Negative	9	75		10	83		8	83		27
		Total	12	100		12	100		12	100		36
	PCR assay	Positive	5	42	0.774 ^{NS}	3	25	0.146 ^{NS}	4	33	0.388^{NS}	12
		Negative	7	58		9	75		8	67		24
		Total	12	100		12	100		12	100		36
Sheep	Microscopic	Positive	4	33	0.388^{NS}	3	25	0.146 ^{NS}	3	25	0.146^{NS}	10
	examination	Negative	8	67		9	75		9	75		26
		Total	12	100		12	100		12	100		36
	PCR assay	Positive	5	42	0.774 ^{NS}	3	25	0.146 ^{NS}	4	33	0.388^{NS}	12
		Negative	7	58		9	75		8	67		24
		Total	12	100		12	100		12	100		36

Abbreviations: NS = Non-significant ($P > 0.05$); S = Significant ($P \leq 0.05$); PCR: Polymerase Chain Reaction.

in Lodhran, and 16.66% (2/12) in Bahawalpur. The overall prevalence across all districts was 19.44% (7/36), statistically non-significant (P=0.06). PCR results revealed a total infection rate of 41.66% (5/12) in Muzaffargarh, 25% (3/12) in Lodhran, and 33.33% (4/12) in Bahawalpur. The overall prevalence across all districts was 33.33% (12/36), also statistically non-significant (P=0.750). For sheep, microscopic examination reported a total infection rate of 33.33% (4/12) in Muzaffargarh, 25% (3/12) in Lodhran, and 25% (3/12) in Bahawalpur. The overall prevalence across all districts was 27.77% (10/36), statistically non-significant (p=0.277). PCR results revealed a total infection rate of 6.30% (1/16) in Muzaffargarh, 0.00% (0/16) in Lodhran, and 0.00% (0/16) in Bahawalpur. The overall prevalence across all districts was 2.10% (1/48), also statistically non-significant (P=0.360) (Tables 1 and 2).

3.5. Binomial test analysis of Babesia spp. infection rates

The binomial test for comparison between positive and negative results across all animals revealed significant differences for microscopic method (P=0.011). Specifically, camel (6/36), cattle (10/36), buffalo (7/36), goat (7/36), and sheep (10/36) showed significant variation. However, through the PCR method, the differences were non-significant (P=0.065), with camels (9/36), cattle (15/36), buffalo (11/36), goat (12/36), and sheep (12/36) displaying no statistically significant variation. In Muzaffargarh, the binomial test for the microscopic method indicated non-significant differences for buffalo (3/12) and goat (3/12) (P=0.146). Through the PCR method, camels (2/12), cattle (6/12), buffalo (5/12), goat (5/12), and sheep (5/12) showed non-significant differences (P=0.774). In Lodhran, the binomial test for the microscopic method revealed significant differences for buffalo (1/12) and sheep (3/12) (P=0.06), while cattle (4/12) showed non-significant differences (P=0.774). Through the PCR method, camels (3/12), cattle (5/12), buffalo (2/12), goat (3/12), and sheep (3/12) displayed highly significant differences (P=0.000). In Bahawalpur, the binomial test for the microscopic method indicated highly significant differences for cattle (2/16), buffalo (1/16), and goat (1/16) (P=0.000), with significant differences for sheep (3/16). Through the PCR method, cattle (0/16), buffalo (0/16), goat (1/16), and sheep (0/16) showed non-significant differences (P=0.146), while camels (2/12) exhibited significant differences (Tables 1 and 2).

4. Discussion

Parasitic diseases, such as babesiosis, exert profound and far-reaching impacts on the global cattle industry, creating a complex web of consequences that extend beyond the immediate concerns of livestock health. These repercussions reverberate across commerce, economies, and even human health, posing formidable challenges to agricultural productivity and economic stability (Omeragic et al., 2022; Chakraborty et al., 2023). Babesiosis, caused by the intraerythrocytic protozoan parasites of the genus *Babesia*, is particularly notorious for its ability to cause anemia, reduce milk production, and compromise the overall well-being of infected cattle (Karshima et al., 2022).

In the context of Southern Punjab, Pakistan, where the cattle industry plays a pivotal role in the livelihoods of many communities, understanding the prevalence of *Babesia* spp. becomes imperative. This study serves as a crucial exploration into the dynamics of babesiosis, utilizing both microscopic and PCR methods for a comprehensive assessment. Microscopic examination allows for the visualization of *Babesia* parasites in blood smears, while PCR, a molecular diagnostic tool, enables the sensitive and specific detection of Babesia DNA, contributing to a more nuanced understanding of the disease landscape in the region.

The intricate interplay between *Babesia* infections and economic factors in the cattle industry underscores the need for targeted interventions and strategic management practices (Heylen et al., 2022). By shedding light on the prevalence of *Babesia* spp. across various livestock species in Southern Punjab, this study not only advances scientific knowledge but also provides a foundation for the development of region-specific strategies to reduce the impact of babesiosis. Ultimately, this research is a crucial step towards fostering sustainable and resilient cattle farming practices in the face of parasitic challenges, benefiting both the agricultural sector and the communities dependent on it.

The study's outcomes underscore the variability in infection rates across different districts of Muzaffargarh, Lodhran, and Bahawalpur in Southern Punjab. Microscopic analysis unveiled babesiosis prevalence rates of 23.33%, 21.67%, and 21.67%, respectively, indicating a notable presence of the parasitic infection. In contrast, PCR analysis presented higher rates of 38.33%, 26.67%, and 33.33% for the same districts, underscoring the heightened sensitivity of PCR in detecting *Babesia* spp. This discrepancy highlights the potential underestimation of prevalence when relying solely on microscopic examination.

The binomial test further elucidated the divergent outcomes between microscopic and PCR methods. Camels, cattle, buffalo, goats, and sheep exhibited significant differences in infection rates through microscopic analysis, emphasizing the varying sensitivity of this method across different animal species. Conversely, PCR results demonstrated statistically non-significant differences in infection rates for camels, highlighting the precision and reliability of PCR in accurately detecting *Babesia* spp. This reinforces the pivotal role of PCR as a preferred diagnostic tool for a more nuanced and accurate assessment of babesiosis prevalence (Mushtaq et al., 2021).

Examining the prevalence across different animal species in Southern Punjab, the study determined that babesiosis follows a distinct order as identified by PCR: cattle > goats > sheep > buffalo > camels. This prevalence pattern is consistent with global studies, underscoring the heightened susceptibility of cattle populations to *Babesia* infections (Jaridehdar et al., 2022; Ali and Marif, 2023; Dhakal, 2023). The findings affirm the pervasive nature of babesiosis, particularly in cattle, across diverse geographical contexts.

The study leveraged PCR as a sensitive and specific diagnostic tool for detecting *Babesia* spp., utilizing GAU primers for DNA amplification. The results demonstrated notable sensitivity, with infection rates of 25.0% in camels, 41.7% in cattle, 30.6% in buffalo, 33.3% in goats, and 33.3% in sheep. These outcomes align with global research, reinforcing the efficacy of PCR in diagnosing *Babesia* spp. infections (Aktaş et al., 2005; Salim et al., 2013; Nugraheni et al., 2023). The sensitivity of GAU primers further emphasizes their utility in enhancing the accuracy of detection.

Regional variation in babesiosis prevalence was evident, with Punjab exhibiting the highest frequency at 33.87%, while KPK recorded the lowest at 13.24%. This regional disparity suggests potential influences from varying veterinary practices and environmental factors, highlighting the need for tailored intervention strategies based on geographical contexts.

The study underscores the crucial role of PCR in conjunction with microscopy for accurate babesiosis diagnosis, emphasizing the method's superior sensitivity and specificity. This emphasizes the pivotal role of advanced molecular techniques in enhancing the precision of diagnostic efforts and informing targeted control measures in the face of babesiosis prevalence.

Regional disparities in babesiosis prevalence, as observed in this study, align with broader scientific evidence. Studies such as those by Bhat et al. (2015), Farooqi et al. (2017a), Bahia et al. (2020), Shoaib et al. (2022) corroborate the notion that geographical variations play a significant role in the prevalence of *Babesia* infections. Specifically, Farooqi et al. (2017b) provide insights into regional prevalence patterns in Pakistan, while Bhat et al. (2015) emphasizes the influence of geographical factors on babesiosis rates. Furthermore, the identified higher frequency in Punjab (33.87%) compared to KPK (13.24%) resonates with the findings of Mushtaq et al. (2021), who discuss regional disparities in parasitic diseases impacting cattle health. Mushtaq et al.'s work in particular delves into the impact of environmental factors on the prevalence of such diseases in different regions. This convergence of findings emphasizes the need for tailored intervention strategies based on the geographical context. By considering regional variations in veterinary practices and environmental factors, interventions can be more precisely targeted, aligning with the recommendations of studies such as Aktaş et al. (2005) which advocate for context-specific approaches to manage parasitic diseases in livestock. The identified regional variation in babesiosis prevalence is well-supported by a body of research, highlighting the importance of acknowledging geographical nuances in developing effective strategies for the prevention and control of this parasitic disease.

The pivotal role of PCR in tandem with microscopy for precise babesiosis diagnosis, as highlighted in this study, aligns with findings from various sources. Notably, Alvarez et al. (2019) emphasize the superiority of PCR as a reference test for *Babesia* species detection, attributing its effectiveness to the method's ability to describe the microorganism at subgenus, species, or even type or strain levels. The significance of advanced molecular techniques, such as PCR, in enhancing diagnostic precision resonates with the broader literature. Study by Hunfeld et al. (2008) further underscore the efficacy of PCR in diagnosing *Babesia* spp. infections, emphasizing its sensitivity and specificity compared to traditional methods.

5. Conclusion

This study underscores the substantial prevalence of *Babesia* spp. in Southern Punjab, Pakistan, with varying infection rates across different districts and livestock species. The comparison between microscopic and PCR methods highlights the precision of molecular assay. The geographic variation in prevalence rates emphasizes the need for region-specific veterinary interventions. The high prevalence of *Babesia* infection in cattle, as revealed by PCR, reinforces the significance of this technique for accurate and species-specific diagnosis. In conclusion, this research contributes valuable insights into the epidemiology of babesiosis, advocating for the routine integration of PCR in diagnostic protocols to enhance sensitivity and reliability.

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