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Prevalence of Babesiosis and Associated Risk Factors in Captive Cervid and Antelope of Punjab, Pakistan

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Key words
Babesia, Molecular Detection, Epidemiology, Phylogenetic Analysis, Cervid, Antelope

ABSTRACT

Captive cervid and antelope play an important role in maintaining the balance of ecosystem but they also serve as a reservoir for wide variety of infections. Tick borne diseases are an issue of major concern for wildlife as well as human populations. Study was specifically designed to generate baseline data about the prevalence, epidemiology, and optimisation of molecular diagnosis of babesiosis in cervid and antelope in captivity under the supervision of the Punjab Wildlife and Parks Department. The study was undertaken with 200 captive wild ungulates' blood samples which were first screened on the basis of microscopic examinations. For molecular diagnosis, whole genomic DNA was extracted and then amplified using 18S rRNA genus based primers. The PCR product was sequenced and phylogenetic tree was established using MEGA11 software neighbour joining method. A total of 30.5% blood samples were found to be positive for babesia by microscopy and out of these microscopic positive, 55.73% were found positive for babesia spp. through PCR and sequencing results showed that our isolate has homology with *Babesia bigemina* strain. Highest positive percentage was found at Safari Zoo Lahore (35.49%) and no positive case was recorded at Bahria Town Zoo, Rawalpindi, Bahawalpur Zoo, Bahawalpur, and Wildlife Park Lohi Bher, Rawalpindi. Among the total PCR positive animals, individually hog deer had highest percentage of positive samples (41.17%). Among the various studied factors, use of acaricides, weather, animal housing area, ticks hidings, vector control programme, quarantine for animals and in and out animal movement were found to be significant risk factors (P<0.05) associated with the occurrence of babesiosis in captive cervid and antelope in Punjab.

INTRODUCTION

Ticks (Ixodidae) play a crucial role in transmitting pathogens, particularly in temperate regions (Jongejan and Uilenberg, 2004). Tick prevalence in wild ungulates may vary significantly depending on the area. More than 300 distinct vertebrate species, as well as free-ranging ruminants (ungulates), are infested by the generalist tick *Ixodes ricinus*. A few of the hemoparasites that ungulates harbour can also afflict people and domestic animals with illness.

The two main blood parasite orders, Piroplasmida and Rickettsia, are transmitted by Ixode ticks among the ungulate species. Piroplasmosis is a widespread disease that primarily affects humans and cattle but also affects many other mammal species. It is rapidly being identified as a public health issue across the board (Zanet *et al*., 2014). Babesia, a frequently found parasite, is transmitted by *Ixodes ricinus* and can infect both domestic and wild animals (Yabsley and Shock, 2013; Sun *et al*., 2014). The

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screening of these ungulates is essential for conducting studies on tick-borne infections and assessing the possible harm to human and animal health due to the function of roe deer as reservoirs of tick-transmitted agents.

The animals suffering from babesiosis exhibit leukocytosis, neutrophilia and lymphedema in their haematology and serum biochemistry as a result of the stress brought on by the disease, liver damage, lesions brought on by the parasite during its blood-borne proliferation, followed by a disruption of liver function, organ necrosis, anorexia-related significant depletion of glycogen reserves is the cause of hypoglycemia, which also disturbs the essential metabolic processes necessary for the animal's normal health and highest level of productivity (Herwaldt *et al*., 1996). Fever, inappetence, and death are the most frequent clinical symptoms seen in TBDs, along with unusual hemoglobinuria and anaemia for bovine babesiosis (Mtshali and Mtshali, 2013).

Clinical symptoms and regular microscopy are the main methods for diagnosing hemoparasites. These conventional diagnostic methods, aside from time-consuming slide scanning, low sensitivity, and a lack of species-level differentiation, are unable to detect the subclinical stage of parasitemia, the cryptic carrier state such as the trophozoite phase in blood, and the carrier stage. Compared to regularly employ traditional parasitological procedures, nucleic acid-based diagnosis, such as PCR, has a number of benefits (Bilgic *et al*., 2017). The recent molecular research has provided support for a variety of studies and contributed fresh, novel perspectives to our understanding of the biology of haemosporidians, particularly with regard to their genetic diversity, phylogeography, phylogeny, and host-specificity for vertebrates (Muieed *et al*., 2010). The protozoal parasites of the genus *Babesia*, which are cosmopolitan in nature and infect a wide variety of captive ungulates, are responsible for the primary tick-borne disease known as babesiosis, which results in significant losses in terms of their high cost and disruptions to natural ecosystem resources. A large number of factors have been recorded in Pakistan that is associated with the spread of *Babesia*. Babesiosis is more common in certain regions, ticks are more active in warmer months so there is association of season also. Factors such as sex, age, adult tick abundance, breed are also important (Ahmad *et al*., 2023; Smith Jr *et al*., 2014; Zia and Nazir, 2019).

The current study was specifically designed to generate baseline data about the prevalence, epidemiology, optimisation of molecular diagnosis and identification of associated risk factors of babesiosis in cervid and antelope in captivity under the supervision of the Punjab Wildlife and Parks Department, Pakistan.

MATERIALS AND METHODS

Sampling site and study area

Keeping in mind the parameters established by the authority for sampling, blood samples were drawn from 200 captive ungulates (cervids and antelopes) being kept at various public and private wildlife parks and zoos in 14 Districts of Punjab, Pakistan (Fig. 1), that displayed one or more typical clinical symptoms of babesiosis as mentioned in inclusion criteria section.

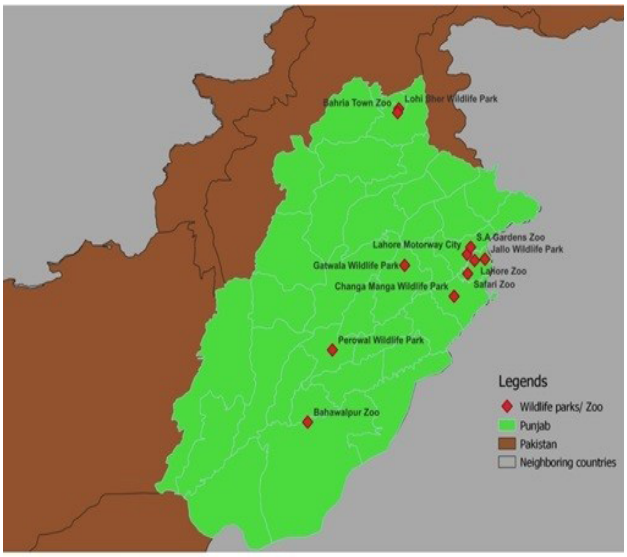

Fig. 1. Sampling districts of Punjab, Pakistan mentioned by red dots.

Animal population

There were 1362 cervids and antelopes present at sampling sites according to stock positions of March 2022 (Table I). Out of these animals, 200 were selected for sampling in order to diagnose *Babesia* and other tick-borne hemoparasites.

The study comprised exhibits that displayed some or all of the following clinical symptoms, such as a history of tick infestation, anaemia, anorexia, weakness, lethargy, and mild to high fever. The information on the sampling site, the animals' species, their management, the environmental conditions, etc., was also recorded.

Sample collection and processing

Aseptic blood samples, obtained using 18-gauge disposable syringes were put into a vacutainer coated with the anticoagulant Ethylene Diamine Tetra Acetic Acid (EDTA). Moreover, thin blood smear slides were also made using aseptic ear tip puncture, and the slides were

dried up on site to be used for microscopy.

The thin blood smears were stained with 10% Giemsa stain after fixation with methanol (Absolute) for 30 sec. and examined under the microscope with 100X oil immersion lens. The samples were stated positive of the presence of inclusion bodies resembling hemoparasites, i.e., *Babesia* were seen (Ullah *et al*., 2022; Soulsby, 1982).

Molecular diagnosis and phylogenetic analysis

All the microscopic positive samples were subjected to molecular diagnosis. DNA was extracted using the GeneAll ExpinTM Combo GP small, 200p kit with Catalog No. 112-102. 40ng of DNA was used as the template for each amplification cycle in a reaction mixture of 20 µl containing 10 µl (2 times) PCR master buffers (Catalog No. W1401-2 wizbio solutions, Korea), 20 pmol of each primer (5'-GTCTTGTAATTGGAATGATGG-3' as forward and 5'-TAGTTTATGGTTAGGACTACG-3' as reverse primer for *Babesia* species self-designed with 465bp size on 18S hypervariable V4 region and 4 µl Diethyl pyro-carbonate (DEPC) treated water (Catalog NO. 750023 INVITROGENTM, USA). The reactions were carried out under the following conditions in a GS482 Thermo cycler (G-STORM, UK).

For the *Babesia* genus, initial denaturation is done at 95°C for 5 min, followed by 30 cycles of 95°C for 1 min and 60°C for 1 min and an extension step for 72°C for 10 min. The amplified products were analysed using electrophoresis on 1.5% agarose gel. Gel electrophoresis was performed on 10 µl of each PCR product at 120 volts for 40 min. Westburg 1kb DNA ladder cat # BR BR0800101 was used to compare the PCR product's size. Primer product with a size of 465 bp was regarded as positive for the genus *Babesia*.

The PCR positive samples for babesia were sent to world renowned laboratory in Singapore for sequencing. Sequencing results were compared with already present sequences at NCBI gene data bank.

Associated risk factors

Along with the prevalence of disease, the suspected risk factors for hemoparasitic infestation, such as species, sex, age, location of the sampling, enclosure type and environment, weather, other disease-potential species in the neighbourhood, in and out animal movements, body condition of the animal, likelihood of ectoparasite infestation, type of skin coat, etc., were also studied with the help of data recorded in data capture performa.

With the use of the site's records, physical traits, examination of tooth replacements, examination of mandibular tooth wear, etc., the animals age were determined. The animals were divided into two groups based on their ages: young (1 year) and adult (> 1 year). The animal's physical state was determined and graded as normal, weak and obese, while taking into consideration its overall condition and estimated body weights (Ali *et al*., 2014).

Statistical analysis

The prevalence was simply determined using descriptive statistics and a percentage formula, and the significance of the related risk factors was assessed using univariate analysis, i.e., Chi-Square test or the Fisher Exact test. The R statistical language version 4.2.2 and its packages "dplyr, lubridate, data table, ordinal, ggplot2 etc." were used to analyse the entire set of data. *P*-value less than 0.05 at the 95% confidence interval was regarded as significant.

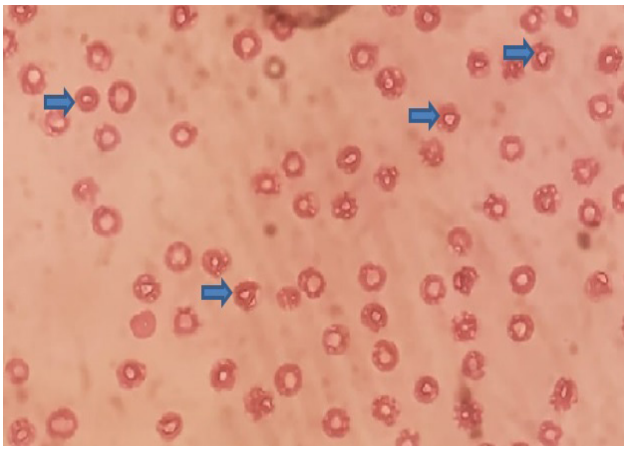

Fig. 2. Rings, pear, oval, round or irregularly shaped intraerythrocytic inclusion bodies (arrows) were seen through thin blood smear at 100X oil immersion lens (Giemsa's staining).

RESULTS

Prevalence of Babesia

The rings, pear, oval, round, or irregular shaped inclusion bodies in RBCs were considered as positive for *Babesia* (Fig. 2) in 30.5% animals. The highest percentage (40.98%) was found in hog deer while lowest in addax, blue bull and fallow deer as 3.27% in each. While 55.73%, among the microscopic positive animals were confirmed positive for *Babesia* species by PCR. Among the total PCR positive animals, hog deer were found highest positive (41.17%) for *Babesia* species while blue bull at lowest percentage as no case was found positive in blue bull (Table I).

Table I. Species and sampling site wise babesia positive cervid and antelope population in Punjab, Pakistan.

Characteristics	Sampled animal (n= 200)	Microscopy positive (n= 61)	PCR positive (n = 34)	p value
Sampling location				0.337
Bahawalpur Zoo Bahawalpur	2	01 (1.63%)	0 (0%)	
Bahria Town Zoo Rawalpindi	7	01 (1.63%)	0 (0%)	
Lahore Motorway City Zoo	14	03 (4.91%)	01 (2.94%)	
Lahore Zoo Lahore	39	11 (18.03%)	06 (17.64%)	
S.A Garden Zoo Sheikhupura	13	03 (4.91%)	02 (5.88%)	
Safari Zoo Lahore	59	17 (27.86%)	12 (35.29%)	
Wildlife Park Changa Manga Kasur	18	08 (13.11%)	05 (14.70%)	
Wildlife Park Gatwala Faisalabad	13	04 (6.55%)	02 (5.88%)	
Wildlife Park Jallo Lahore	24	09 (14.75%)	04 (11.76%)	
Wildlife Park Lohi Bher Rawalpindi	3	0 (0%)	0 (0%)	
Wildlife Park Perowal Sahiwal	8	04 (6.55%)	02 (5.88%)	
Sampled Animal species				0.490
Addax	3	02 (3.27%)	01 (2.94%)	
Black buck	15	04 (6.55%)	02 (5.88%)	
Blue bull	3	02 (3.27%)	0 (0%)	
Chinkara	10	03 (4.91%)	01 (2.94%)	
Fallow deer	3	02 (3.27%)	01 (2.94%)	
Hog deer	95	25 (40.98%)	14 (41.17%)	
Mouflon sheep	44	13 (21.31%)	07 (20.58%)	
Punjab urial	10	03 (4.91%)	02 (5.88%)	
Samber deer	6	03 (4.91%)	02 (5.88%)	
Spotted deer	11	04 (6.55%)	04 (11.76%)	

The highest molecular positive percentage of disease (35.29%) among the various locations was found at Safari Zoo Lahore while lowest (i.e., no positive case was recorded) at Bahria Town Zoo, Rawalpindi, Bahawalpur Zoo, Bahawalpur, and Wildlife Park Lohi Bher, Rawalpindi (Table I). Moreover, sampling location (p=0.337) and animal species (p=0.490) were not found significant factors in univariate and multivariate analysis towards disease occurrence.

The representative sample sequence (OR512079.1) >1st_BASE_4869120_Babesia_F showed 100% similarity with *Babesia bigemina.* Phylogenetic tree represented relationship between inter-species along with genetic characterisation; it demonstrated the divergence across fully or partially interconnected species and their evolutionary relationship. The evolutionary relationships between closely related species and other related species were clearly seen in the distance tree data in Figure 3.

Fig. 3. Phylogenetic relationship of *Babesia* based on *18S rRNA* gene. All accession numbers correspond to different species of Babesia and Theileria. The sequences generated in the present study is named as 1st BASE 4869120 Babesia F.

Table II. Risk factors associated with occurrence of babesiosis in cervid and antelope of Punjab, Pakistan.

Characteristics	Levels	Positive	Negative	p-Value
Age of animal	Young (≤ 1 Year)	01(2.94%)	19 (11.45%)	0.13
	Adult (>1years)	33 (97.05%)	147 (88.55%)	
Sex of animal	Female	19 (55.88%)	86 (51.80%)	0.66
	Male	15 (44.12%)	80 (48.19%)	
Diet of animal	Green fodder	04 (11.76%)	25 (15.06%)	0.69
	Green fodder plus concentrate	27 (79.42%)	120 (72.29%)	
	Pelleted feed	03 (8.82%)	21 (12.65%)	
Presence of ticks	Present	02 (5.88%)	06 (3.61%)	0.54
	Not present	32 (94.12%)	160 (96.38%)	
Use of acaricides	No	30 (88.23%)	119 (71.69%)	0.043
	Yes	04 (11.76%)	47 (28.31%)	
History of T.B.D	No	02 (5.88%)	16 (9.63%)	0.49
	Yes	32 (94.12%)	150 (90.36%)	
Treatment for T.B.D	No	6 (17.64%)	33 (19.87%)	0.76
	Yes	28 (82.35%)	133 (80.12%)	
Weather/Season	Summer	12 (35.29%)	50 (30.12%)	0.01
	Fall	5 (14.70%)	40 (24.10%)	
	Spring	11 (32.35%)	21 (12.65%)	
	Winter	6 (17.64%)	55 (33.13%)	
Water for drinking	Stagnant	4 (11.76%)	08 (4.81%)	0.12
	Tab	30 (88.23%)	158 (95.18%)	
Other species around	Bovines	16 (47.05%)	68 (40.96%)	0.61
	Caprines	6 (17.64%)	22 (13.25%)	
	Equines	01 (2.94%)	09 (5.42%)	
	None	11 (32.35%)	67 (40.36%)	
Animal housing area	Large cage	17 (50%)	65 (39.16%)	0.04
	Medium cage	10 (29.41%)	30 (18.07%)	
	Small cage	07 (20.58%)	71 (42.77%)	
Cracks/Tick hidings	Not present	06 (17.64%)	59 (35.54%)	0.04
	Present	28 (82.35%)	107 (64.46%)	
Vector control program	Not practiced	30 (88.23%)	117 (70.48%)	0.03
	Practiced	04 (11.76%)	49 (29.52%)	
Quarantine for animals	No	24 (70.58%)	86 (51.81%)	0.04
	Yes	10 (29.41%)	80 (48.19%)	
Body condition	Normal	20 (58.82%)	98 (59.04%)	0.51
	Obese	2 (5.88%)	20 (12.05%)	
	Weak	12 (35.29%)	48 (28.91%)	
Skin coat type	Thick and hairy	31 (91.17%)	149 (89.75%)	0.80
	Thin and less hairy	03 (8.82%)	17 (10.24%)	
In and out animal movement	Frequent	24 (70.58%)	78 (46.99%)	0.03
	Less frequent	06 (17.64%)	40 (24.10%)	
	No movements	04 (11.76%)	48 (28.91%)	

Associated risk factors

Table II shows the various risk factors associated with occurrence of babesia infection in captive cervid and antelope of Punjab, Pakistan. The captive sites where acaricides were being used inside enclosures were having very low babesia infections. The p-value 0.043 was showing significance of the factor in disease occurrence. The season was also found a substantial risk factor with p=0.01 in occurrence of babesiosis. In summer season highest positive percentage was recorded. The type and size of housing area was found a significant factor for occurrence of babesia infection with p=0.04. Majority of the positive animals for babesiosis were from those enclosures where hiding sites like cracks were present. Statistically the role of cracks or tick hiding places in the enclosure was also found significant (p=0.04) in univariate analysis. The vector control program was also found a significant risk factor towards disease occurrence with p=0.03. The positive percentage of vector control program at non-practicing sites was higher. The captive sites where quarantine protocol was not practiced showed higher positive percentage and the factor was found having significant role (p=0.04) in disease occurrence. While the in-and-out movements of animals at captive sites were discovered to be a significant (p= 0.03) risk factor for the occurrence of babesia infection, with a high positive percentage at sites where frequent movements of animals were present

Tap water was being used in large number of positive animals with p=0.12 which was showing no relation with disease occurrence. As far as the age of sampling animals was concerned, adult animals were having more positive percentage and statistically, it was also found that age was not having significant role (p=0.13) in occurrence of disease. Similarly, the gender was also found not to be a serious risk (p=0.66) for babesia infection. The analysis of data regarding the type of diet offered to the animals at captive sites showed no role in disease occurrence with p=0.69. The presence of ticks was found a non-significant factor (p=0.54) in causing babesia infection in captive cervid and antelope. Whereas, the history of tick borne diseases (TBD) was not found a significant factor in disease occurrence with p=0.49 along with the factor, previous treatment of tick borne diseases (p=0.76). The other tick host species around the captive sites as grazers were found to be a non-significant (p=0.61) in occurrence of disease. The body condition and skin coat type of animals were found non-significant risk factors for occurrence of babesia infection with p-values 0.51 and 0.80, respectively (Table II).

DISCUSSION

Babesia spp. that spread by ticks can cause significant economic damage by infecting the erythrocytes of a variety of vertebrates (Khetran *et al*., 2019). *Babesia* cause severe diseases in wild and domestic animals (Kuttler *et al*., 1988). Compared to other traditional approaches (forming blood smears and doing serological tests), DNA amplification by PCR is a sensitive and specific tool for the diagnosis of babesiosis (Aktas *et al*., 2005; Nagore *et al*., 2004). Given that it is a component of the ribosomal functional core and is subject to identical selective pressures in all living things, the small subunit 18S rRNA gene is one of the most crucial markers for the PCR-based identification of a number of parasites, including *Babesia* spp. (Criado-Fornelio *et al*., 2003). We believe that our study will provide a thorough assessment of the epidemiology, genetic basis for diagnosis, and factors associated for babesiosis in captive cervid and antelope in Punjab, Pakistan. It was found that molecular identification of *Babesia* spp. in captive cervid and antelope was accurate and less ambiguous with high positive percentage. Although blood parasite infections in many wildlife species are typically asymptomatic, clinical signs can develop under certain conditions such as unnatural hosts, stress, habitat degradation, climate fluctuation, or immunosuppression (Williams *et al*., 2014).

The early detection of blood parasites is vital. Traditionally, the primary method for identifying babesia and theileria organisms in infected animals has been microscopic examination using Giemsa-stained blood smears, especially in acute cases. However, this approach falls short in efficiently detecting carriers with low levels of parasites, where only a few protozoa exist in the peripheral blood (Bose *et al*., 1995). To overcome this limitation, serological techniques have been proposed to identify circulating antibodies against these parasites, particularly in subclinical infections during epidemiological studies. The indirect fluorescent antibody test is an example of a method used to detect antibodies against theileria species (Leemans *et al*., 1997). Nevertheless, these serological tests have drawbacks, including generating false-positive and false-negative outcomes due to cross-reactions or improper specific immune responses (Passos *et al*., 1998). Furthermore, they cannot differentiate between past and current infections, necessitating the use of sensitive and highly specific diagnostic techniques for babesia and theileria. Consequently, the use of PCR-based techniques has become essential for detecting these hemoparasites in carrier animals (Criado-Fornelio *et al*., 2009). These PCR-based approaches have been utilized for diagnosing babesiosis and theileriosis in various species in countries with similar climatic conditions, such as Tunisia (Mghirbi

et al., 2008), the United Arab Emirates (Jaffar *et al*., 2010), and Iran (Zaeemi *et al*., 2011). According to gender-specific data presented by (Ali *et al*., 2014) males died most frequently 23.71% from parasite infestation, followed by trauma 20.62% while, among the parasites babesiosis and theileriosis were also identified 5.07%.

Michel's findings indicate that as altitude increases, the number of ticks' decreases. Notably, there's a distinct discrepancy in tick-borne infections between young and adult roe deer. Young roe deer, or kids, are more frequently infected with either *B. capreoli* or *Babesia* sp. EU1 compared to adult deer, as described in Michel's research. In the case of Holstein Friesian (HF) cattle kept in tethered housing systems without supplementary feed during the summer season, the prevalence of babesiosis and theileriosis was found to be statistically significant ($P < 0.05$) in young cattle (> 16 months). Similarly, the prevalence of babesia and theileria in another breed of cattle (presumably WB) at a young age and housed in tethered housing systems during the summer season also showed significant statistical importance ($P < 0.05$), according to the study (Zaman *et al*., 2022).

The impact of climate change extends to the transmission of infectious diseases, where vectors like ticks play a significant role in affecting both animals and humans (Altizer *et al*., 2013). Ticks rely on the environment, climate conditions, and hosts for their survival, with climate change being a key factor in their distribution and the spread of tick-borne pathogens (Medlock *et al*., 2013). This phenomenon is particularly pronounced at northern latitudes and altitudes, as demonstrated in the case of *Ixodes ricinus* and *Borrelia burgdorferi sensu lato* (Dantas-Torres, 2010).

Capture and confinement stress can lead to the reactivation of latent infections, as shown in instances like the occurrence of fatal equine piroplasmosis caused by *B. equi* in newly bred horses (Dennig, 1966). In wild animals, transmission of *Babesia* spp. from domestic animals can have lethal consequences, indicating the presence of *Babesia* spp. in the wild with varying host specificity, maintaining a state of endemic stability. Stressors like capture and temporary captivity can trigger clinical babesiosis in wild animals, even from typically non-harmful parasites, as observed in black rhinos (Penzhorn, 2006).

The higher occurrence of infections in enclosures with short vegetation can be attributed to the creation of tick-friendly environments characterized by shrubs and grass. Additionally, there is supporting evidence indicating a positive correlation between the infection rates of hosts and the density of tall shrubs and undergrowth, especially concerning *Borrelia burgdorferi.* Another contributing factor to the expansion of endemic babesia populations is the presence of neighbouring or co-housed cervid species.

CONCLUSION

Babesia spp. was detected in 30.5% of captive cervid and antelope in Punjab, Pakistan, by microscopy. Of them, 55.73% were confirmed to be positive by PCR. Numerous risk factors, including the vector control program, the animal housing area, cracks and tick hiding places, quarantine practices and the inside and outside movement of animals have been proven to be significant in the occurrence of disease.

RECOMMENDATIONS

To control the disease in captive cervid and antelope, multi-faceted approach is needed to achieve the required goals. Appropriate screening, quarantine practices, tick control, veterinary monitoring and treatment protocols may be used to prevent significant losses in the form of mortality in the subject animals. For the fulfilment of true objective of domestic wildlife conservation, the education, training and research initiatives might potentially be expanded to all over Pakistan in collaboration with other professional stake holders and institutions.

DECLARATIONS

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Ethical/ IRB approval

No animals were harmed throughout the study, which was conducted in accordance with Institutional Review Committee vide letter no. DR/402 dated 30 September 2021 issued from University of Veterinary and Animal Sciences Lahore, Pakistan.

Declaration of generative AI and AI-assisted technologies

It is declared with honesty that no generative AI and AI-assisted technologies have been used in write up process. The whole manuscript was self-written manually.

Statement of conflict of interest

The authors have declared no conflict of interest.

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