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Article

# Development and Validation of a Novel Multiplex PCR Assay for the Detection of *Anaplasma*, *Babesia*, and *Theileria* in Captive Wild Ungulates

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**Abstract:** Wildlife animals have integral significance in ecological and economic stability. They are becoming extinct. One of the key roots of endangerment is tick-borne diseases. *Anaplasma*, *Babesia*, and *Theileria* are major parasites transmitted by obligatory hematophagous arthropods known as ticks to wildlife, livestock, and humans. In the current study, the creation of novel primers and optimization of a multiplex PCR assay to detect hemoparasites were done. Blood samples were collected from a total of 60 captive *Gazella bennettii*, family Bovidae, and *Axis porcinus*, family Cervidae, from different wildlife parks and zoos in Punjab, Pakistan. Both families were targeted during sampling to increase the sample count and obtain a variety of ungulates. Microscopy was performed using the Giemsa staining method, and 40 samples tested positive, which were then proceeded with PCR. Total genomic DNA was extracted for molecular detection by PCR. This study comprised three sets of newly designed primers based on genus to optimize multiplex PCR for melting temperature and crosslinking among primers. The results of our multiplex PCR assay were completely consistent with monoplex PCR using newly designed primers. Results of multiplex PCR revealed the presence of total hemoparasitic species infection in 65% of samples, whereas individual infections of *Anaplasma*, *Babesia*, and *Theileria* were recorded as 25%, 0%, and 40%, respectively. PCR provided accurate results supported by sequencing. This multiplex PCR assay provides a valuable complementary tool in routine, simultaneous, early, and accurate detection of *Anaplasma*, *Babesia*, and *Theileria* genera that helps control antibiotic resistance and save endangered species.

**Keywords:** *Gazella bennettii*; *Axis porcinus*; anaplasma; babesia; theileria; multiplex PCR

## Introduction

Wildlife is now considered an important source and reservoir of a wide range of parasites around the globe, which is also a great threat to wildlife itself and human populations. Parasite flow across wildlife may occur through a variety of routes, one of which is an arthropod vector (Polley, 2005). Anaplasmosis, babesiosis, and theileriosis are a group of tick-borne diseases caused by protozoan parasites and have a great impact on tropical and subtropical ungulates such as hog deer (*Axis porcinus*) and chinkara (*Gazella bennettii*) in Pakistan (Demessie and Derso 2015). These parasites

have an economic impact on both livestock and wildlife. The infected sporozoite stage of *Anaplasma*, *Babesia*, and *Theileria* is transmitted through the saliva of different tick species like *Rhipicephalus* and *Boophilus* as they feed on a host. *Anaplasma* targets WBCs, *Babesia* primarily targets RBCs, while *Theileria* successively targets WBCs and RBCs. (Ibrahim & Sander, 2022).

Overall, in blood-borne diseases, clinical signs and symptoms observed are fever, anaemia, anorexia, constipation, dyspnoea, dehydration, weight loss, poor milk supply, diminished performance, jaundice, sunken eyes, rapid abdominal respiration, severe lung involvement, emaciation, lacrimation, corneal opacity, and frothy nasal discharge (Mattioli et al. 1994); (Buriro et al. 1994); (Naz et al. 2012).

Accurate detection of these parasites is essential for effective disease management, as it can be difficult to differentiate between them when making a diagnosis. Misdiagnosis of disease leads to morbidity and mortality. Diagnostic tests for blood parasites, such as Microscopy, enzyme-linked immunosorbent assay (ELISA) and immunofluorescence antibody assay (IFAT), and RDTs (Rapid diagnostic test), give false negative results, and no intra-species differentiation is seen (Berzosa et al. 2018). To identify pathogens in the early phase of disease, a new diagnostic test based on DNA is essential for rapid diagnosis and proper treatment.

Multiplex PCR detects multiple targets in a single reaction well using a distinct pair of primers for each target. It is time and cost effective, especially for detecting mixed infections capable of detecting multiple parasites at various stages of infection at the same time (Edwards and Gibbs 1994). Successful diagnosis of various bacterial, viral, fungal and parasitic infections using multiplex PCR has been reported (Markoulatos et al. 2002).

In the current study, we optimized a multiplex PCR assay targeting the internal transcribed spacer (ITS) regions of *Anaplasma*, *Babesia*, and *Theileria* for their detection, as these parasites invade the circulatory system of a host and cause mortality by damaging blood cells. Accurate diagnosis and timely treatment of such diseases, especially in endangered species, is crucial. In wildlife, obtaining samples is a complicated task, particularly in cases of mixed infections. Conducting individual disease tests can lead to a loss of valuable samples and time. This study aims to develop an assay that would be useful for the timely diagnosis of co-infections in a single reaction, which will be very useful in wild animal medicine, where repeated sampling is a major issue.

## Materials and Methods

### *Blood Sample Collection*

A total of 60 blood samples were collected from the Chinkara and Hog deer being kept at various public and private wildlife parks and zoos in 14 Districts of Punjab, Pakistan, that displayed one or more typical clinical indications of anaplasmosis, babesiosis, and theileriosis were taken by using common minimally invasive capture methods. The animals were not specifically captured for research purposes; rather, samples were collected opportunistically during routine captures conducted for medical examinations and other scheduled procedures. Positive blood samples from captive animals comprising Chinkara and Hog Deer were further processed. Blood samples were taken from the jugular vein in EDTA vials for the best preservation of cellular components and the morphology of blood cells. Samples were collected by qualified veterinarians as per the standard sample collection method without any undue stress to animals. The blood samples were stored at 20°C until DNA extraction.

Microscopy

Thin blood smears were made from all samples and stained with 10% Giemsa stain after fixation with methanol (Absolute) for 30 seconds. Samples were initially screened based on microscopic examination under a 100X oil immersion lens. The specimens were stated to be positive upon the presence of inclusion bodies resembling hemoparasites i.e., *Anaplasma*, *Babesia* and *Theileria*.

Primer Designing for rDNA Intergenic Spacer Region

For Deoxyribonucleic acid extraction of blood samples phenol-chloroform extraction protocol was used. The blood was lysed with lysis buffer to get white pellets, which was further treated with phenol chloroform isoamyl alcohol to get nucleotides, specifically DNA. The extracted total DNA was visualized by gel electrophoresis using Bio-Helix Novel juice as DNA staining reagent. DNA fragments of different lengths were seen. A Nano-Drop 2000/2000c spectrophotometer (Thermo Scientific, USA) was used to check the concentration and purity of extracted DNA.

The genus-based primers were designed by aligning sequences of 16S rRNA of *Anaplasma* species and 18S rRNA of different *Babesia* and *Theileria* species at Mega3 software. Various *Theileria* Species, *T. buffeli*, *T. sergenti*, *T. cervi*, *T. annulata* and *T. parva* with accession number of sequences AB000272.1, AB016074.1, AY735122.1, KF429795.1, L02366.1 respectively, those of *Babesia* Species *B. ovata*, *B. ovis*, *B. divergens*, *B. odocoilei*, and *B. gibsoni* with accession number of sequences XR\_003751973.1, AY260178.1, U07885.1, U16369.2, OQ727056.1, respectively and those of *Anaplasma* species, *A. ovis*, *A. Phagocytophilum*, *A. bovis*, *A. Centrale* and *A. Playts* with accession number of sequences AY262124.1, AB196721.1, AB211163.1, AB211164.21, EU439943.1, respectively were retrieved from NCBI. These primers were further checked by Primer Blast for their specificity for each parasite. Primer sequences and their amplicon sizes are shown in Table 1. The specificity of primers was bioinformatically aligned with databases in the National Center for Biotechnology Information (NCBI).

**Table 1.** Primer sequences, targeted genes used for *Anaplasma*, *Babesia* and *Theileria* in the current studies.

Target  organism	Genetic  marker	Primer Sequence (5' to 3')	Prim  er  lengt  h	T  m  (°C  )	GC  %	Amplic  on Size  (bp)
Anaplasmatac  eae	16sRNA	5'AGACGGGTGAGTAATGCA	19	62.	47.4	318
	Ana_F	T3'		7		
	16sRNA	5'AAGAGTTTTACAACCCTA	22	62.	40.9	
	Ana_R	AGGC3'		3		

Babesia spp.	18sRNABab _F	5'GCTCTTTCTTGATTCTTTGG GT3'	22	62. 9	40.9	479
	18sRNABab _R	5'TAGGCAAAACCGACGAAT C3'	19	62	47.4	
Theileria spp.	18sRNA Thl_F	5'GCTCTTTCTTGATTCTTTGG GT3'	22	62. 9	40.9	148
	18sRNA Thl_R	5'CTCTAAGAAGCGATAACG GG3'	20	61. 6	50	

### PCR Amplification

The PCR assay was standardised using extracted genomic DNA obtained from samples that were tested as a positive control for each parasite by sequencing of monoplex PCR amplicons. PCR primer sets were validated separately to determine their specificity. PCR was optimized to ensure appropriate amplification. The multiplex PCR assay was developed using these newly designed primers in consideration of the size of the PCR product and annealing temperature. The final 35 µl PCR mixture comprised of 5 µl of DNA extract from each positive sample, 2µl of forward and reverse primer (10 pm/ml) from each of the *Anaplasma*, *Babesia* and *Theileria*, 1 µl Taq DNA Polymerase, 1.5 µl MgSO<sub>4</sub>, 2.5 µl buffer and 3 µl nuclease free water. Amplification was carried out using the following cycling conditions: initial denaturation at 94°C for 5 min followed by 10 cycles of 94°C for 30s, 45 s at 65°C (Decreasing 1°C per cycle), at 72°C for 1 min, followed by 30 cycles at 94°C for 30s, 45s at 55°C, at 72°C for 1 min and final extension at 72°C for 10 min. Samples were held at 4°C before analysis. Each reaction product was visualized using a UV trans illuminator after staining with ethidium bromide.

### Sensitivity and Specificity

The sensitivity of the multiplex primer assays (Ana\_F/Ana\_R, Bab\_F/Bab\_R, and Thl\_F/Thl\_R) was assessed by employing serial dilutions of DNA templates ( $1 \times 10^2$  –  $6 \times 10^2$  ng) with strains utilized as positive controls. Specificity was assessed by sequencing.

## Results

### Microscopy

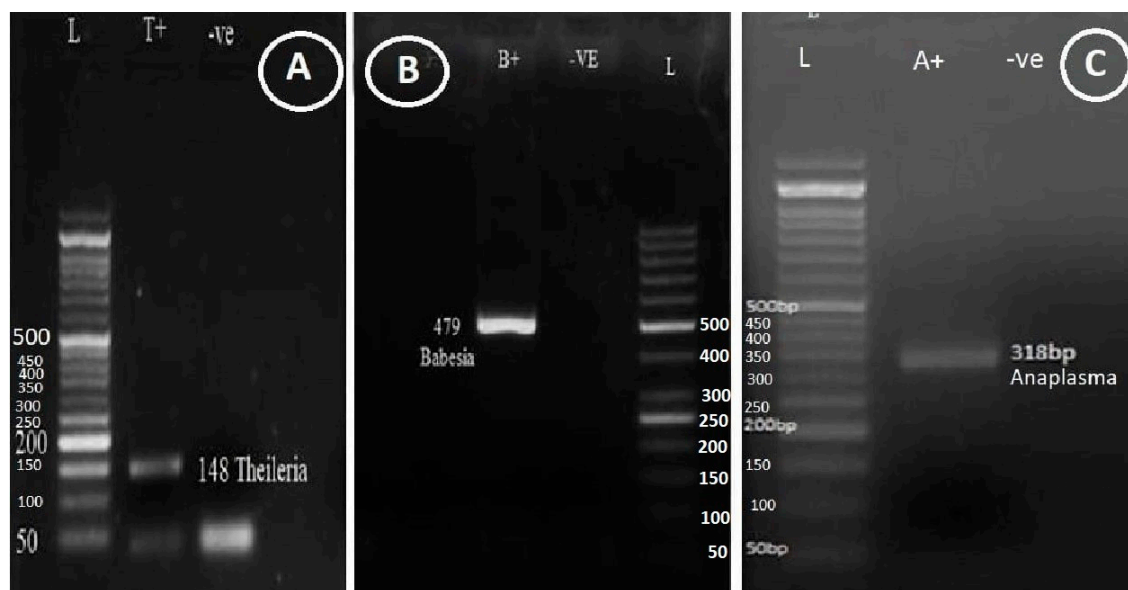
Microscopy of 60 samples was performed, out of which 40 showed a positive result for parasitic presence. Single infection was observed in 35 (58.33%) samples, including 9 (15%) with *Anaplasma*, 4



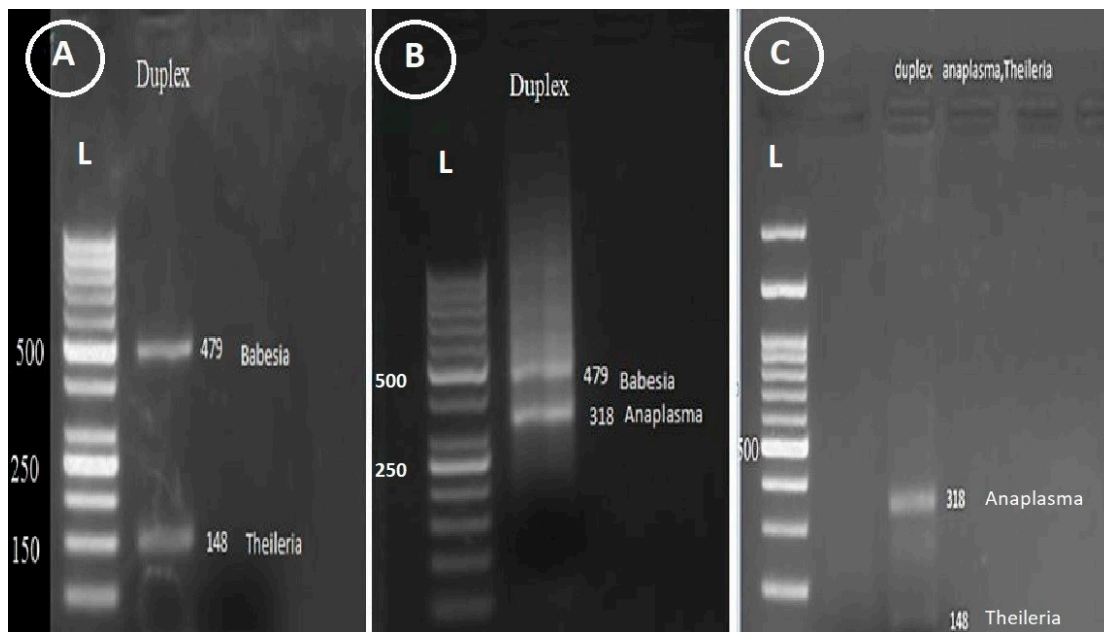
(6.6%) with *Babesia*, and 22 (36.7%) with *Theileria*. While 5 (8.3%) samples showed mixed infection, i.e., positive for more than one parasite at a time in a single sample.

#### Multiplex PCR (mPCR) Standardization

The multiplex PCR (mPCR) assay was optimized using genomic DNA extracted from confirmed positive blood samples. These positive samples were validated through sequencing of monoplex PCR amplicons, each corresponding to a specific band length characteristic of the target protozoa. After analysing the results of monoplex PCR assay, duplex and triplex PCR (Figures 1–3) setups were created by combining specific oligonucleotide sets in different ways, effectively amplifying all relevant markers.



**Figure 1.** Detection of *Theileria*, *Babesia* and *Anaplasma* using monoplex PCR. (A) *Theileria* 148-bp (T+ positive control, -ve negative control, Lane L; BIOLADDER 50-bp DNA ladder), (B) *Babesia*, 479-bp (B+ positive control, -ve control, Lane L; GB-Ruler 50-bp DNA ladder) and (C) *Anaplasma*, 318-bp and Lane L; BIOLADDER 50-bp DNA ladder.



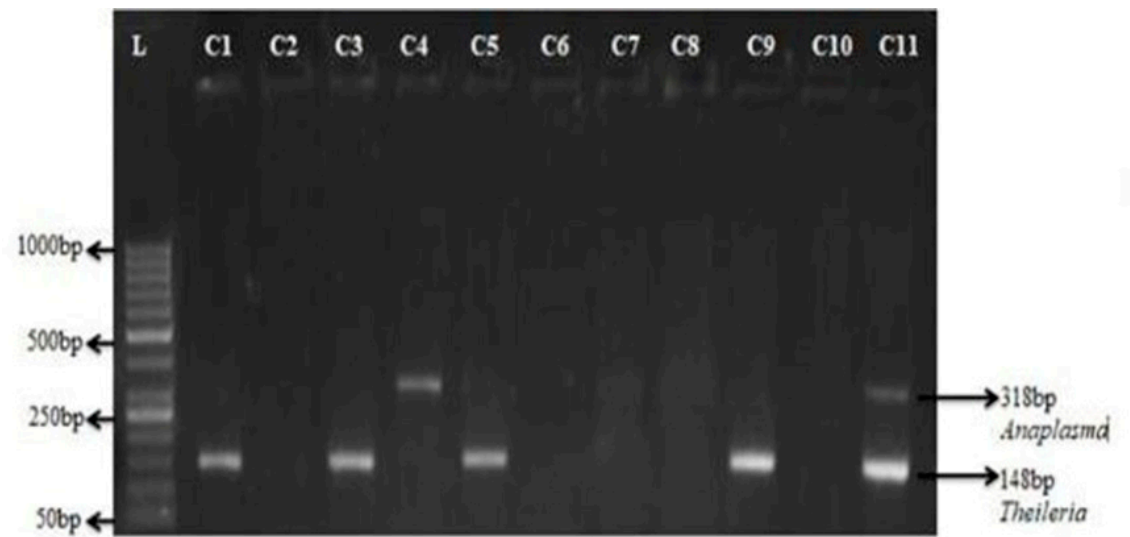
**Figure 2.** Agarose gel electrophoresis of amplified DNA from different *Theileria*, *Babesia* and *Anaplasma* species using combination of primers in duplex fashion (A) *Babesia* spp. and *Theileria* spp., (B) *Anaplasma* spp. and *Babesia* spp., (C) *Anaplasma* spp. and *Theileria* spp. Specific amplicons for *Theileria* 148-bp, *Babesia* 479-bp, and *Anaplasma* 318-bp approximately. Lanes marked L: GB-Ruler 50-bp Molecular weight marker.



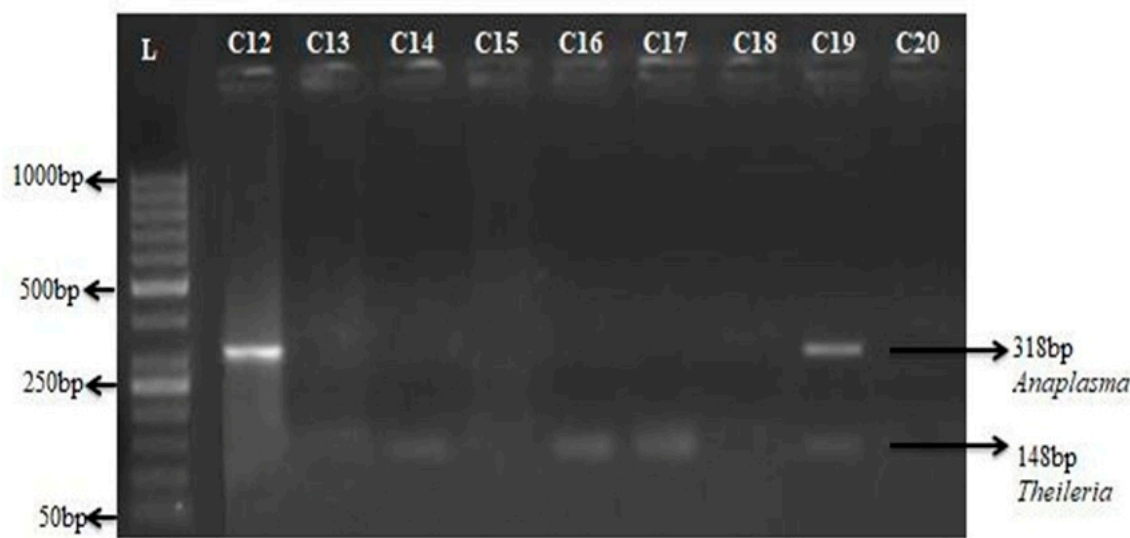
**Figure 3.** Agarose gel electrophoresis of amplified DNA from different *Theileria*, *Babesia* and *Anaplasma* species using multiplex PCR. (PCR products of *Babesia* 479bp, *Theileria* 148bp and *Anaplasma* 318bp). Lane L: GB-Ruler 50-bp molecular weight marker.

Performance of Multiplex PCR Assay

Developed Multiplex PCR Assay was performed on all microscopically screened field samples. Overall prevalence rate of all the pathogens was found 65% among the microscopy positive samples including 25%, 0% and 40% individually of *Anaplasma*, *Babesia* and *Theileria*, respectively (Figures 4–7).

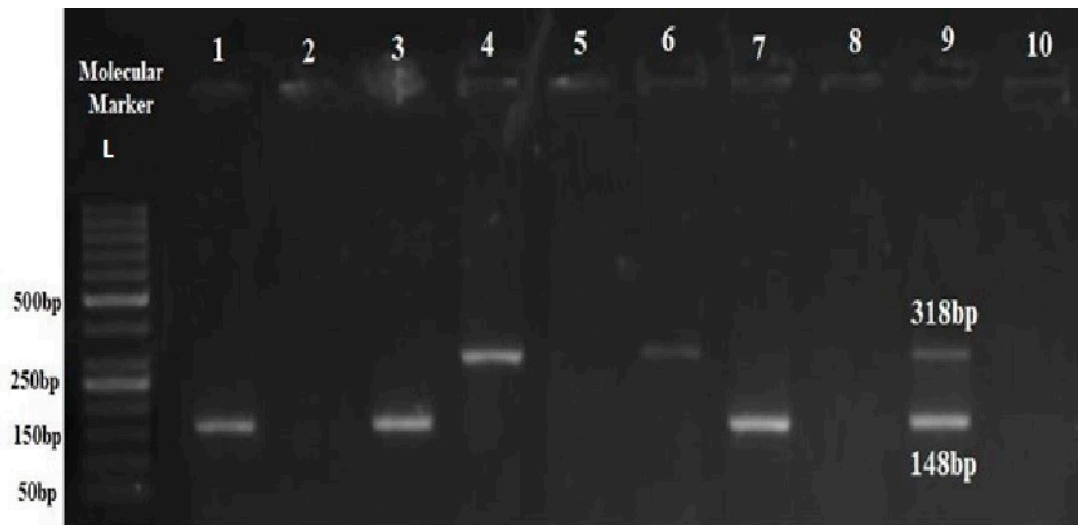


**Figure 4.** Detection of *Anaplasma* in field samples using multiplex PCR. Agarose gel electrophoresis of amplified DNA from different *Theileria* and *Anaplasma* species using multiplex PCR on the Field samples of chinkara C1-C11. (PCR products *Theileria* 148bp and *Anaplasma* 318bp Lane L: 50 bp marker).

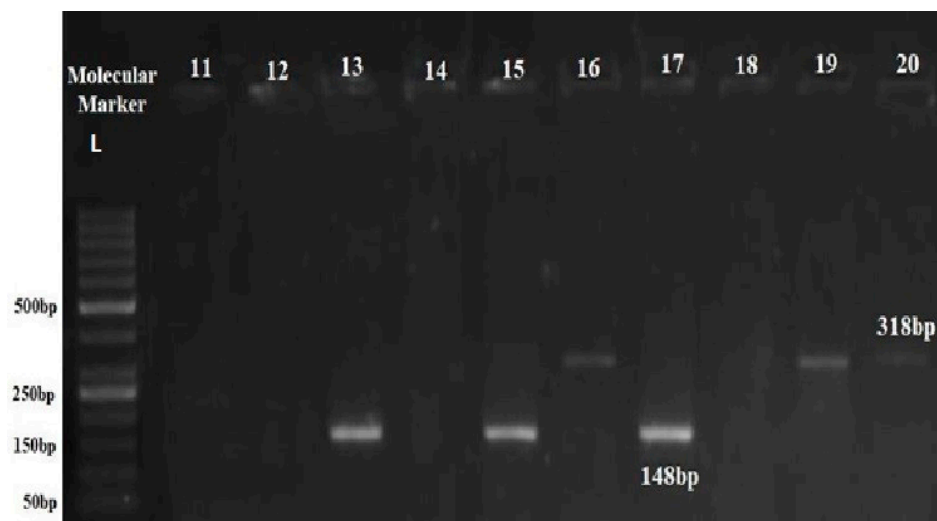


**Figure 5.** Detection of *Anaplasma* in field samples of chinkara using multiplex PCR. Agarose gel electrophoresis of amplified DNA from different *Theileria* and *Anaplasma* species using multiplex PCR on the Field samples of chinkara C1-C11. (PCR products *Theileria* 148bp and *Anaplasma* 318bp Lane L: 50 bp marker).





**Figure 6. Detection of *Anaplasma* in field samples of Hog deer using multiplex PCR.** Agarose gel electrophoresis of amplified DNA from different *Theileria* and *Anaplasma* species using multiplex PCR on the Field samples of Hog deer C1-C11. (PCR products *Theileria* 148bp and *Anaplasma* 318bp Lane L: 50 bp marker).



**Figure 7. Detection of *Anaplasma* in field samples of Hog deer using multiplex PCR.** Agarose gel electrophoresis of amplified DNA from different *Theileria* and *Anaplasma* species using multiplex PCR on the Field samples of Hog deer C11-C20. (PCR products *Theileria* 148bp and *Anaplasma* 318bp Lane L: 50 bp marker).

#### Sensitivity

The mPCR results indicated a sensitivity level of  $3 \times 10^2$  ng, establishing the method as sensitive for detecting DNA of *Anaplasma*, *Babesia*, and *Theileria* in all multiplex combinations. Every primer pair utilized in triplex PCR assays was designed to target a specific gene, and their distinct sizes were differentiated through gel electrophoresis for each of the three infectious pathogens.

#### Validation of Primer Specificity by Sequencing

Sequencing was done on representative positive Controls. Using BLAST (Basic Local Alignment Search Tool) and MEGA (version 5.05), the acquired sequences were compared to sequences already present in the GenBank database, validating the specificity of the designed primers.

The sequences from all three positive parasite controls were submitted to GenBank, and accession numbers were obtained as OR610413 (*Anaplasma*), OR512079 (*Babesia*), and PP455035 (*Theileria*).

## Discussion

The prevalence and importance of tick-borne diseases have been examined worldwide (de la Fuente et al. 2023, Han et al. 2009, Ahmad et al. 2008). Changes in climate and a reduction in environmental diversity have led to an increase in tick-borne disease infections (Seong et al. 2015, Dantas-Torres F. 2015). Hemoprotozoan species such as *Anaplasma*, *Babesia*, and *Theileria* pose significant risks to the health of animals, which include both livestock and wildlife, as well as humans (Maharana et al. 2016). For example, some research indicates that infection rates of *Babesia* and/or *Theileria* were found to be 89.7% (156 out of 174) in roe deer in Spain (Remesar et al. 2019), 58.3% in brown brocket deer (*Mazama gouazoubira*) and marsh deer (*Blastocerus dichotomus*) in Brazil (Da Silva et al. 2011), and 35.3% in sika deer (*Cervus nippon*) in China (Liu et al. 2016). Likewise, *Theileria* is also notably prevalent in various wild and domestic animals, with studies revealing an infection rate of 4.7% in wild boars, 22.2% in Alpine chamois, and 1.0% in red foxes in Italy (Zanet et al. 2014); 80.0% in cattle, 93.8% in sheep, and 1.9% (5 out of 265) in goats, with a total of 235 out of 525 (44.8%) ruminants testing positive for *Theileria* in Ethiopia (Gebrekidan et al. 2013); and 6.2% (82 out of 1329) in dogs in South Africa (Matjila et al. 2008). In Korea, as in other nations, ticks are widespread. Numerous studies on tick-borne diseases have been undertaken involving cattle, dogs, and horses (Seong et al. 2015). The infection rates were 1.2% (9 out of 737) in cattle (Kwak et al. 2020), 3.1% (16 out of 510) in dogs (Seo et al. 2020), and 0.9% (2 out of 224) in horses (Seo et al. 2013).

Economic losses have been seen due to high morbidity and mortality rates in small ungulates. This is the first study designed for the molecular detection of three important hemoprotozoan in wild ungulates kept at different wildlife parks of Punjab and comparison of molecular diagnosis with typical microscopy. Multiplex PCR assay was developed focusing on ITS region of *Anaplasma*, *Babesia* and *Theileria* to detect these parasites simultaneously in a single reaction tube. Detection of these parasites is not only crucial for protecting wildlife but also provides epidemiological links and prevalence rates of parasites.

High prevalence rate of *Theileria* infection shows multiple/repeated exposure to the infection throughout an animal's whole life. In northwest Poland, 90% of red deer were found positive for *theileria* (Sawczuk et al. 2008) and also in Korean water deer, *Theileria* found to be the highly infectious pathogen (Seong et al. 2015). Current studies show *Theileria* is more prevalent compared to *Anaplasma* and *Babesia*. In this study, the prevalence of *Anaplasma* spp. as provided by blood smear microscopy is 27.5% which is in accordance with the studies on prevalence of *Anaplasma* spp. in buffalos and cattle in Pakistan (Buriro et al. 1994; Rajput et al. 2005). Multiplex PCR assay provided 30% *Anaplasma* positive samples. Molecular detection-based studies have also provided prevalence rates of *Anaplasma* spp. 32% and 33% in Asia (Kordick et al. 1999; Motoi et al. 2001).

This analysis suggests that deer are not exposed to *Babesia* as it is not detected even in one out of forty blood samples. It might be due to the small number of samples. In Spain, nearly 90% of roe deer were found to carry either *Babesia* or *Theileria* piroplasm, with *Theileria* spp. showing a higher prevalence of 60.9% compared to *Babesia* spp. at 19.0%. In 17.3% of positive samples, the specific species could not be identified (Remesar et al. 2019). In a Turkish study on distinct horse sub-populations, 18.50% of samples tested positive for *Babesia* and *Theileria* infections using cELISA. *T. equi* had a significantly higher prevalence (16.21%) compared to *Babesia* (0.83%) among the examined blood samples. (Sevinc et al. 2008). The trees generated propose that *Babesia* and *Theileria* are closely related, originating from a paraphyletic group (Allsopp et al. 1994). The intimate phylogenetic affinity between *Theileria* and *Babesia* (Aktas et al. 2002) renders their differentiation via microscopy a formidable task due to this there is difference between microscopy and PCR results in present study. In Kouhdasht, Lorestan Province, PCR testing targeting the 18s-rRNA gene indicated a 4.7% infection rate of *B. ovis* in sheep and goats, while microscopic examination revealed a significantly higher 12.2%

rate for *Babesia*, attributed to the microscope's inability to distinguish between *Babesia* and *Theileria* (Naderi et al. 2017).

The internal transcribed spacer (ITS) region is a crucial component of the ribosomal RNA (rRNA) gene complex, and it plays a significant role in molecular biology, particularly in the fields of genetics, genomics, and phylogenetic Analysis. They have a high degree of variability between species and are used to identify species. ITS2 is a vital marker in molecular systematics and serves in species barcoding and DNA array technologies (Engelmann et al. 2009). It can differentiate closely related species, so it is useful in detecting co-infection in single host (Blouin 2002; Li and Yang 2008). It is a key challenge to find conserved regions and design primers targeting all the species.

Multiplex PCR is the co-amplification of multiple targets, so it depends upon the compatibility of primers used, particularly the  $T_m$  adjusted to anneal the specific targets at the same time (Exner 2012). All the primer sets are designed to target the amplicon of distinct size, so that fragments of desired length can be seen easily by agarose gel. This technique's genetic markers enable discrimination between closely related species or strains (Mahoney and Chernesky 1995). It has now been recognized as a rapid and reliable detection tool in clinical and research laboratories because considerable time, effort and cost can be saved by amplifying multiple targets in a single reaction. Successful assay development requires step by step optimization and multiple attempts to adjust concentrations of primers and nucleic acids (Markoulatos et al. 2002). Multiplex PCR assay is developed to overcome the problem of false positive results obtained by microscopy which lead to treatment and cause parasites drug-resistance polymorphism (Cui et al. 2015).

This study can be applied to large sample size to small ungulates to better understand the prevalence of the infections. These infections not only affect the health of animals but also affect the food products harnessed from livestock. This will conserve important wildlife and livestock that are important parts of the economy of our country. Epidemiological studies regarding infections and preventive measures to control the spread of infections will be developed.

## Conclusions

This study provided prevalence rates of various parasites *Anaplasma* (25%), *Babesia* (0%) and *Theileria* (40%) by mPCR. Novel mPCR assay development will contribute a significant advancement in veterinary diagnostics. Future studies may expand its application in diverse ecological settings, further aiding in the prevention and control of vector-borne diseases. Overall, this research will help in addressing emerging health challenges in wildlife populations.

## Recommendations

It is recommended that multiplex PCR should be used to detect various infections in a single run to save time, and money. Large scale epidemiological study is the need of the hour to protect wildlife.

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