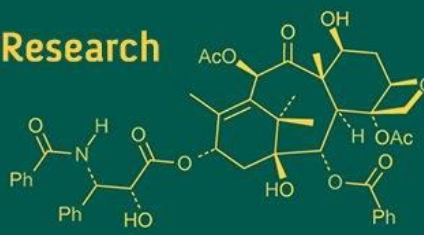
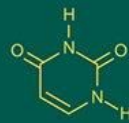


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
IJABR 2025; SP-9(7): 321-329
www.biochemjournal.com
Received: 01-06-2025
Accepted: 03-07-2025

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Molecular prevalence and phylogenetic traits of *Babesia bigemina* in cattle (*Bos taurus*) from north Gujarat of India

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DOI: <https://www.doi.org/10.33545/26174693.2025.v9.i7Se.4826>

Abstract

Bovine babesiosis is a tick-borne disease caused by haemo-protozoan parasites of the genus *Babesia* with four main species viz., *Babesia bigemina*, *Babesia bovis*, *Babesia major* and *Babesia divergens* that cause bovine babesiosis. Ticks mostly present in the study area are *Rhipicephalus (Boophilus) microplus*, *Hyalomma anatolicum anatolicum* etc. Our study highlighted the need for comprehensive studies focusing on the prevalence of babesiosis in North Gujarat and emphasizes the significance of molecular characterization of *Babesia* spp. for designing effective control strategies. A total of 685 blood samples of cattle's were collected from different organized farms/Gausalas/Pantrapoles of Banaskantha district of Gujarat. Blood samples were examined using blood smear and Polymerase Chain Reaction assay to estimate the prevalence of babesiosis according to breed, age and season. The overall prevalence of *Babesia* infection in cattle was found to be 18.54% by microscopy and 27.00% by PCR, demonstrating the greater sensitivity of PCR as contrast to blood smear analysis. No blood samples were found to be positive for other *Babesia* spp. in microscopic as well as in PCR based assay. Breedwise, HF crossbred cattle showed (32.30%) higher prevalence than indigenous Kankrej cattle (19.46%). Babesiosis was found to be more common in age group of 3-5 years old followed by 1-3 and > 5 years old and calves below < 1 year showed the least infection. Summer had highest prevalence, followed by monsoon and winter season. Based on phylogenetic analysis, the *Babesia* spp. sequences (KU SKN CAE *B. bigemina*, KU SKN CAB *B. bigemina*, KU SKN CA28 *B. bigemina*, KU SKN CA29 *B. bigemina*) displayed identity matches with other sequences. This study offers insightful information about the prevalence of babesiosis in various age groups, species, and seasons of bovines, which can help with the implementation of various strategies depending on the specific condition being treated and effective therapeutic measures, vector control policies and suitable preventive and control measures.

Keywords: Bovine babesiosis, North Gujarat, Kankrej cattle, *Hyalomma*, PCR

Introduction

A tick-borne illness that affects both domestic and wild mammals is babesiosis, which is mainly caused by *Babesia* spp. Cattle tick *Rhipicephalus microplus* is the main vector of bovine babesiosis, which causes severe economic losses (Suarez and Noh, 2011; Azhar *et al.*, 2023) [5, 22]. It causes fever, anaemia, haemoglobinuria, jaundice, loss of appetite, trembling in the muscles, and even death. Cattle in India are frequently infected with *Babesia bigemina*, which can lead to severe symptoms such as fever, elevated heart and respiration rates, decreased appetite and haemolytic anaemia. In untreated cases, the disease can result up to 30% of deaths (Kumar *et al.*, 2016) [14]. Low parasite counts and the limitations of conventional microscopic techniques make it difficult to detect carrier and subclinical infections (Sharma *et al.*, 2016) [21]. Preventing the risk of spread of disease need early identification and treatment for which the best and most accurate techniques for identifying these infections are molecular techniques, like nucleic acid amplification (Bose *et al.*, 1995) [6]. This study was aimed to investigate the *Babesia* spp. of cattle in conventional as well as molecular techniques.

Material and Methods

Ethical approval: The experimental procedures received approval from the Institutional Animal Ethics Committee (IAEC) of Veterinary College, Sardarkrushinagar, on January 9th, 2022. The IAEC no. assigned to this approval was VETCOLL/IAEC/2022/19/Protocol-22

Blood collection

Random blood samples were collected from various farms, Gausalas, Panjrapoles, and veterinary clinics of Banaskatha district of North Gujarat. Blood were collected in 5 ml EDTA vacutainer from total 685 cattle of which 298 were Kankrej cattle and 387 were HF crossbred cattle. Before blood collection, cattle were thoroughly examined for tick infestations, disease indicators, and clinical symptoms.

Parasitological method

From the collected blood samples, thin blood smears were made, air dried, fixed in methanol for 5 min and stained with working dilution of 10% Giemsa stain for 30-45 min and washed with tap water, air dried and examined under oil immersion for demonstration of *Babesia* piroplasms, if any.

DNA Extraction and Sequencing

With a few minor adjustments, genomic DNA was extracted from 200 µl of cow blood using the QIAamp® DNA Blood Mini Kit (Thermo Scientific, Lithuania; Qiagen, Germany) according to the manufacturer's instructions. With a ratio of 1.7 to 2, the optical densities at 260 and 280 nm were compared to determine the concentration and purity of the extracted DNA using a spectrophotometer (Eppendorf, Hamburg, Germany). The extracted genomic DNA was then stored at -20°C for further analysis.

Genus and species-specific PCR targeting the *Rap1-C* (Bbig278F/R) fragment of *B. bigemina* (Bhat *et al.*, 2015) was performed using primers synthesized by Eurofins Biotech, Bangaluru. The primer sequences were: *B. bigemina*: Forward 5'-CAT CTA ATT TCT CTC CAT ACC CCT CC-3'; Reverse 5'-CCT CGG CTT CAA CTC TGA TGC CAA AG-3' and *B. bovis*: Forward 5'-CAC GAG GAA GGA ACT ACC GAT GTT GA-3'; Reverse 5'-CCA AGG AGC TTC AAC GTA CGA GGT CA-3'. The 25 µl PCR reaction mixture included 12.5 µl of the 2X Dream Taq Green PCR master mix, 2 µl of forward and reverse primers, 2 µl of DNA template, and 8.5 µl of nuclease-free water. First denaturation at 94°C for 5 minutes was followed by 35 cycles of denaturation at 94°C for 45 seconds. Annealing was done at 57°C for 45 seconds (for *B. bigemina*) or 55°C

for 30 seconds (for *B. bovis*) and extension at 72° C for 45 seconds and the final extension was done at 72°C for 4 minutes for 35 cycles. To determine the expected size of amplicons specific to the targeted organism, amplified DNA fragments were subjected to electrophoresis on a 1.2% agarose gel and visualised using a gel documentation system (Life Technologies, USA).

To confirm specific amplification, the amplified DNA sequences of *Babesia bigemina* were sent to Eurofins Genomics India Pvt. Ltd., Bengaluru, for bi-directional Sanger sequencing. The 278 bp *Babesia bigemina* product's identity was confirmed through the nBLAST tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). A 100 bp DNA ladder was used to run 2 µl of the PCR result on a 1% agarose gel in order to verify integrity. 10 µl of each primer and 28 µl of the PCR product were sent for sequencing. The sequences were compared to those in GenBank using the BLASTn tool (NCBI, USA), MEGA X, and MegaAlign (DNASTar, USA) for pairwise and phylogenetic distance studies.

Results and Discussion

Overall Prevalence of *Babesia* spp.

Out of 685 total blood samples, 127 (18.54%) showed positive results for *Babesia* spp. using Giemsa staining, whereas 183 (26.71%) showed positive results using PCR, indicating higher sensitivity of PCR than the gold standard microscopy. Although Kumar *et al.*, 2016^[14] observed a greater frequency in south-western Gujarat, most likely as a result of sampling from clinically suspicious animals, our data are consistent with those of earlier research (Harish *et al.*, 2006; Vahora *et al.*, 2012; Maharana *et al.*, 2016)^[9, 16, 25]. Our PCR results agree with previous research (Khan *et al.*, 2020; Nugraheni *et al.*, 2023; Vidhya *et al.*, 2023; Mohanta *et al.*, 2023; Najar *et al.*, 2024)^[13, 17, 18, 19, 26]. Variations in environmental conditions, animal populations, disease control tactics, sample techniques, and management practices contributed to the increased prevalence in our study. Additionally, the abundance of the vector *R. microplus* may also be a role.

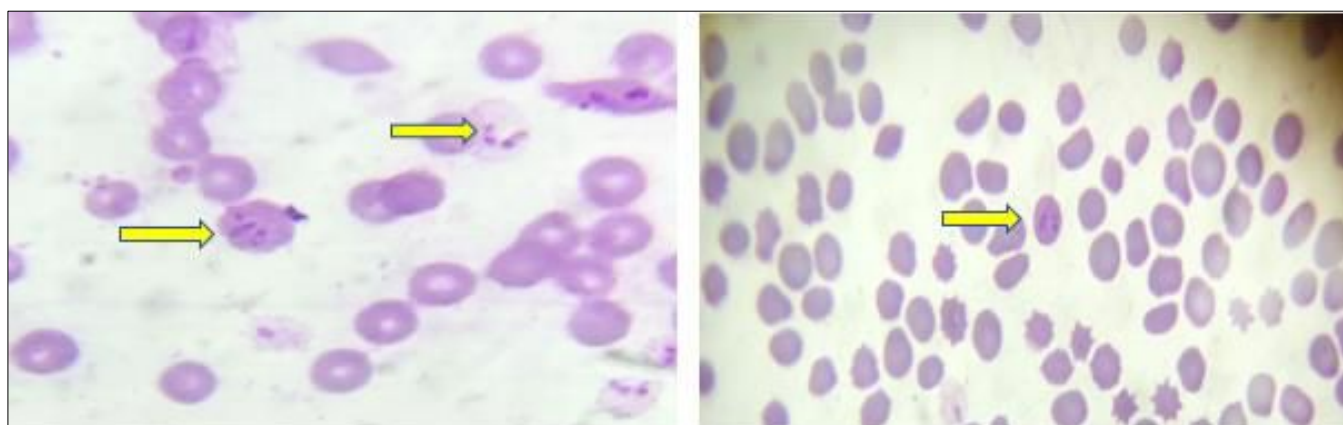


Fig 1: *B. bigemina* within infected RBCs under Giemsa staining

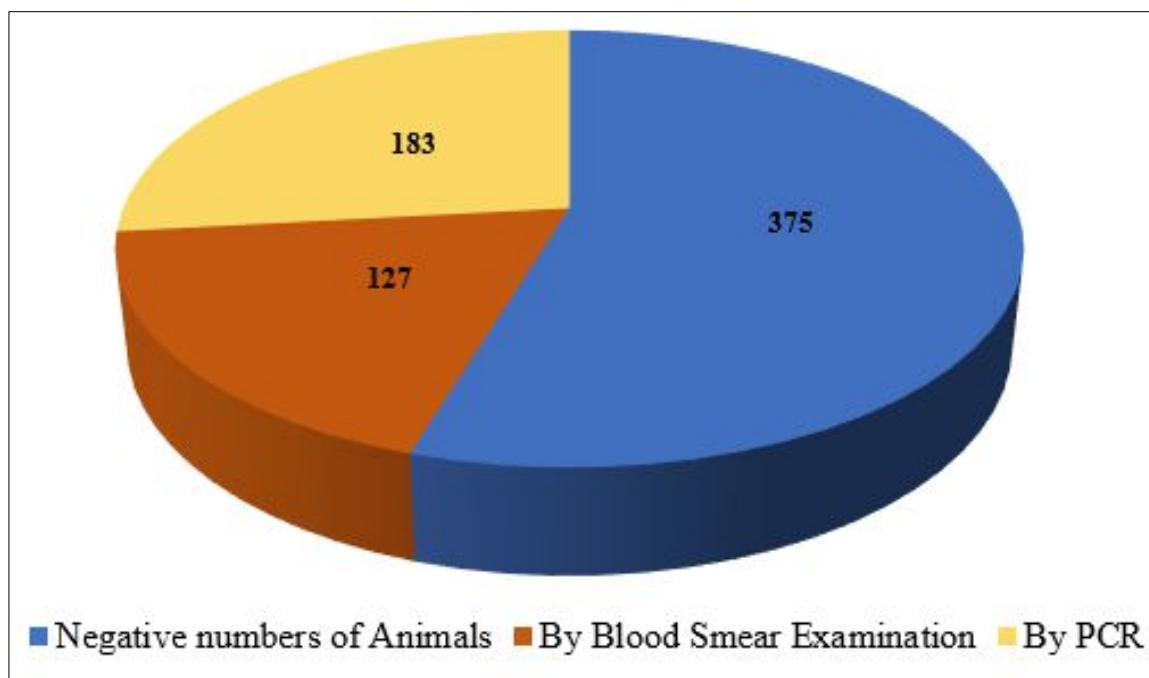


Fig 2: Overall Prevalence of *Babesia* spp. in cattle

Species-wise *Babesia bigemina* infection in Cattle

Table 1: Species-wise *Babesia bigemina* infection in Cattle.

Animal Species	Animal Breed	No. of Samples	By blood Smear Examination	By PCR
Cattle	Kankrej	298	33 (11.07%)	58 (19.46%)
	HF cross bred	387	94 (24.29%)	125 (32.30%)
	Total	685	127 (18.54)	183 (26.71)

In this investigation, the prevalence of *Babesia* spp. in Kankrej and HF cross cattle was 11.07% and 24.29% by microscopy, and 19.46% and 32.30% by PCR, respectively. Compared to crossbred cattle, the prevalence of *Babesia* spp. was lower in Kankrej (11.07% microscopy, 19.46% PCR). These findings are consistent with those of Tariq *et al.*, 2024^[23], who found that prevalence rates in cattle were 21.3% by microscopy and 32.4% by PCR. This greater prevalence of babesiosis in crossbred as compared to

indigenous cattle was also found by Kaur *et al.*, 2021; Al-Shammari *et al.*, 2024^[3, 12]. This discrepancy might be due to genetic differences that lack resistance genes crossbred cattle which increases the susceptibility of tick infestations. These results may be explained by variables including temperature, ecology, tick species, sample gathering techniques, high tick populations, and warm, humid environments that encourage the growth of ticks and piroplasm.

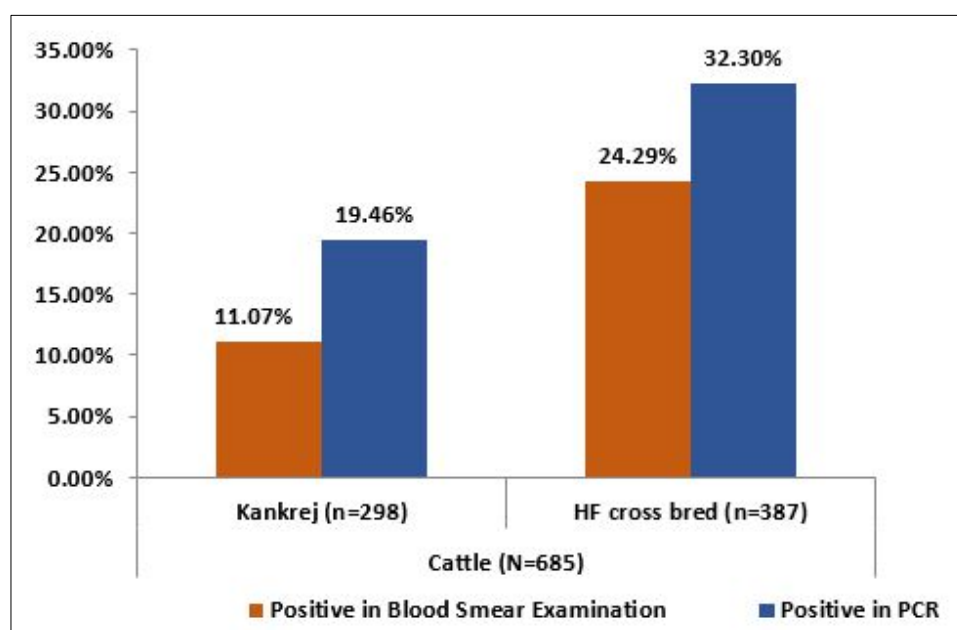


Fig 3: Prevalence of *Babesia bigemina* according to species

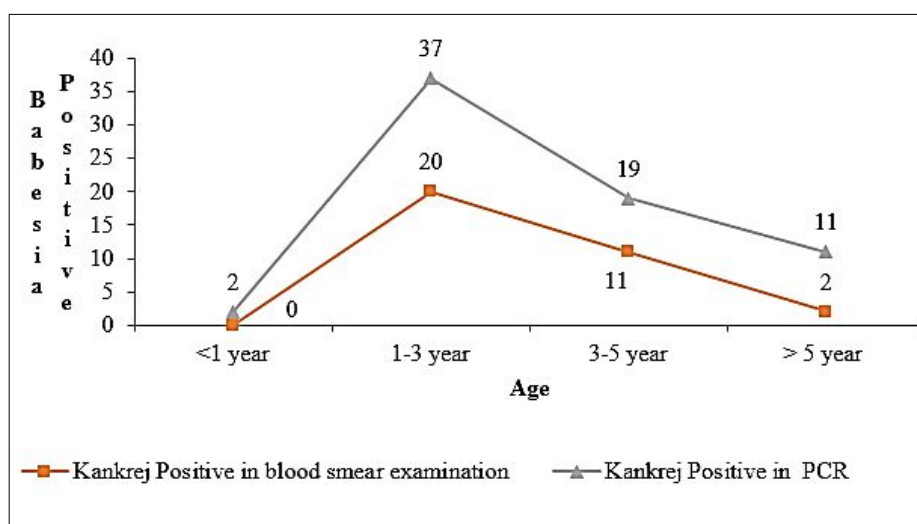
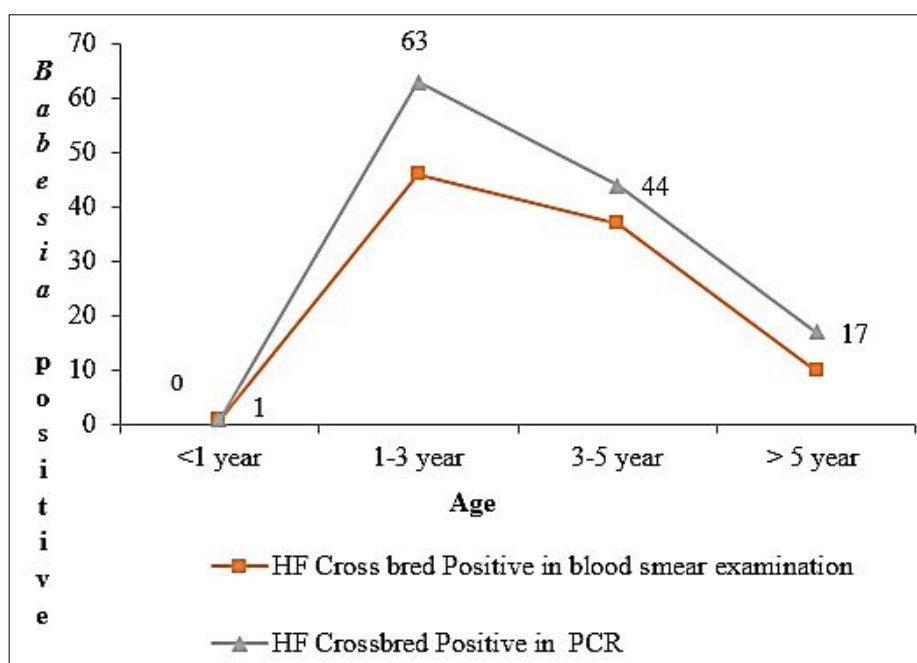
Age wise *Babesia bigemina* infection in cattle

Babesiosis prevalence in Kankrej and HF cattle was highest in the 3-<5 year age group (33.14%) in this study. Rates were lowest in the 1-<3 year group (3.42%) and much animals over 5 years (5.55%). According to PCR data, the prevalence of HF (39.29%) and Kankrej (24.83%) was greater in the 3 to 5-year age group and 1-to-3-year age group, respectively. Because of their age resilience, no illnesses were discovered in extremely young animals. Animals older than five years and calves less than one year showed lower prevalence. In calves younger than a year old,

babesiosis was uncommon (9.52% for Kankrej and 3.12% for HF). Research conducted by (Zaman *et al.*, 2022) [27] likewise found that older animals had greater prevalence rates, with adults having the highest rates. The prevalence was lowest in the physiologically non-active (not mating, not pregnant, not breastfeeding) group and highest in the immature group, (Ola-Fadunsin *et al.*, 2021) [20]. Babesiosis is more common in older animals; adult lactating cattle are especially susceptible, probably as a result of prolactin hormone and genetic factors (Farooqi *et al.*, 2017) [8].

Table 2: Age wise *Babesia bigemina* infection in cattle

	Kankrej			HF Crossbred		
	(n=)	Microscopically	By PCR	(n=)	Microscopically	By PCR
<1 year	21	00	02 (9.52%)	32	00	01 (3.12%)
1-<3 year	149	20 (13.42%)	37 (24.83%)	166	46 (27.71%)	63 (37.95%)
3-<5 year	92	11 (11.96%)	16 (17.39%)	112	37 (33.04%)	44 (39.29%)
>5 year	36	2 (5.55%)	03 (8.33%)	77	11 (14.28%)	17 (22.08%)
Overall	298	33 (11.07%)	58 (19.46%)	387	94 (24.28%)	125 (32.29%)

**Fig 4:** Prevalence of babesiosis in Kankrej according to age**Fig 5:** Prevalence of babesiosis in HF Crossbred according to age

Calves have softer skin and are therefore more susceptible to tick attachment, even if they are initially resistant due to passive immunity from colostrum (James *et al.*, 1988) [11] and a robust innate immunological response (Zintl *et al.*, 2005) [29]. They continue to be vulnerable as their immune systems develop and their passive immunity diminishes (Zia *et al.*, 2019) [28]. Due to immunological memory and higher reactions to re-exposure, older animals that have been exposed to the parasite for a longer period of time have stronger immune defences (Zia *et al.*, 2019) [28].

Season wise *Babesia bigemina* infection in cattle

In this study, summer showed the highest prevalence of babesiosis, followed by monsoon and the lowest prevalence was found in the winter in both microscopy and PCR. Microscopic diagnosis revealed the summer prevalence with 66.67% in Kankrej and 70.21% in HF Crossbred, 27.27% and 19.94% were found in monsoon and 6.06% and 10.63% in the winter. PCR technique revealed the higher summer prevalence with 51.72% and 60.8%, 32.76%, 32.76% and 27.2% in the monsoon and 15.51% and 12.00% in the winter.

Table 3: Season wise *Babesia bigemina* infection in cattle

		Total no of samples collected	Winter	Summer	Monsoon	
Total cattle sample collected		685	112	326	247	
PCR Positive	Kankrej	58	09 (15.51%)	30 (51.72%)	19 (32.76%)	
	HF crossbred	125	15 (12%)	76 (60.8%)	34 (27.2%)	
Microscopic positive	Kankrej	33	02 (6.06%)	22 (66.67%)	09 (27.27%)	
	HF crossbred	94	10 (10.63%)	66 (70.21%)	18 (19.14%)	
Total buffaloes sample collected		223		40	120	63
PCR Positive buffaloes		22		00 (0%)	16 (72.73%)	6 (27.27%)
Microscopic positive		15		00 (0%)	11 (73.33%)	4 (26.67%)

Our studies were convenient with the findings of Zaman *et al.*, 2022; Ali *et al.*, 2022; Ahmad *et al.*, 2023; Dhakal *et al.*, 2023, Ullah *et al.*, 2023 [1, 2, 7, 24, 27] where summer showed the highest prevalence ranging from 25% to 50%. This is

because of the suitable higher environmental temperature for tick reproduction in summer which increases the incidence.

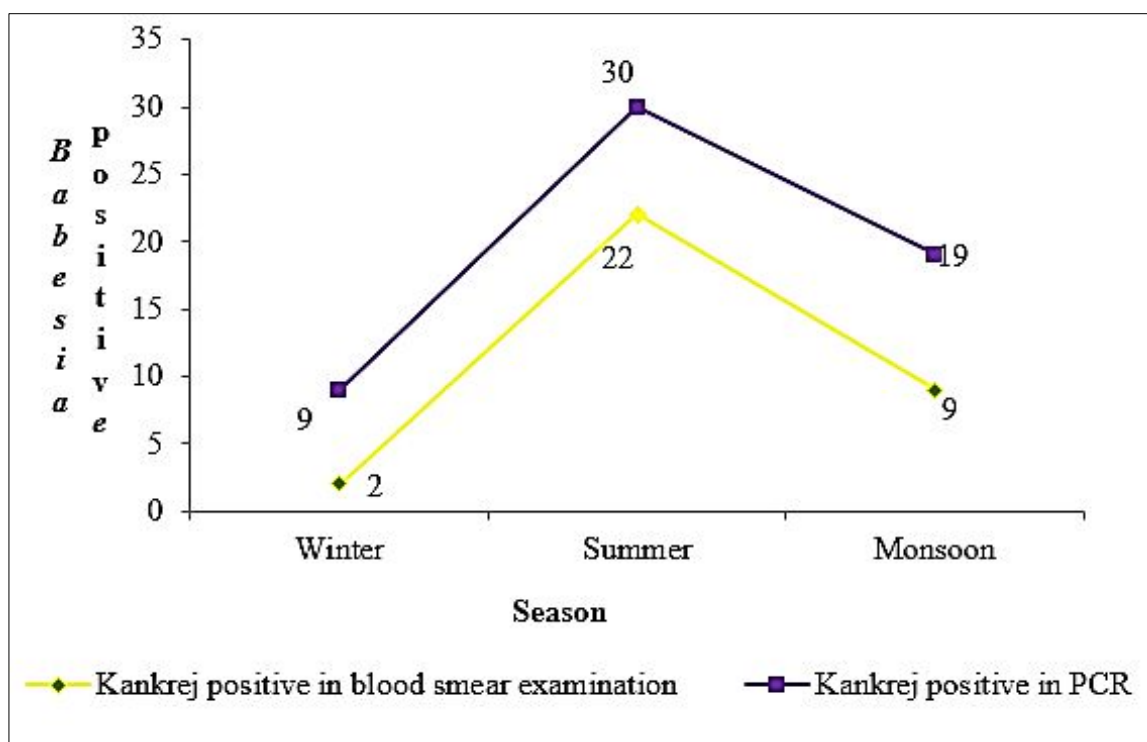


Fig 6: Prevalence of babesiosis in Kankrej cattle according to season

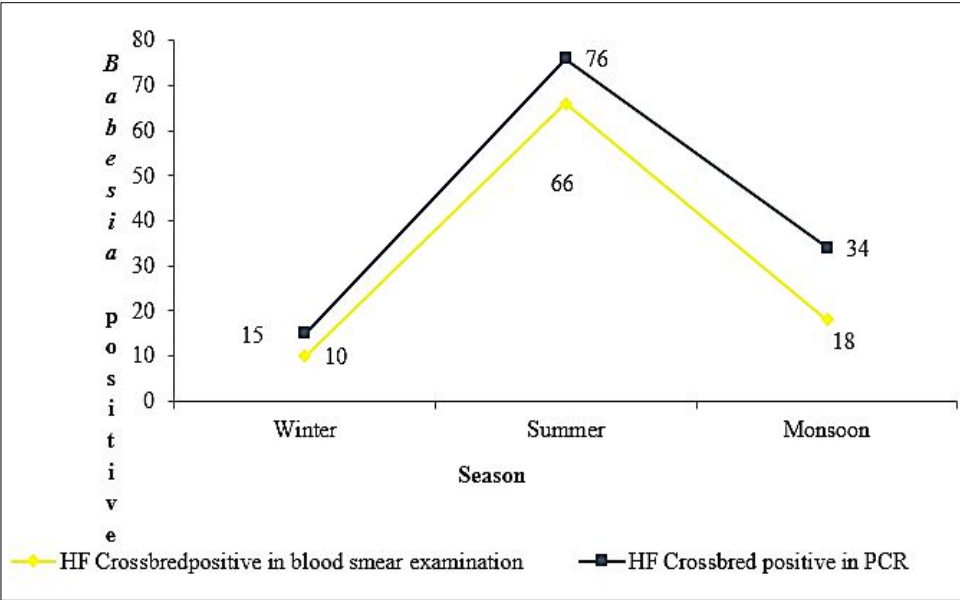


Fig 7: Prevalence of babesiosis in HF Crossbred according to season

Molecular Detection of *Babesia* spp. in cattle

In molecular detection, *B. bigemina* was found in 19.46% of Kankrej cattle and 32.30% of HF crossbred cattle. No positive samples for *B. bovis* were found. These findings are found consistent with other studies by Mohanta *et al.*, 2023; Najar *et al.*, 2024 [17, 18] with a prevalence of 33.62% in Kashmir, and Tariq *et al.*, 2024 [23] with a prevalence of

32.4% in Pakistan. Tick susceptibility may be related to the higher occurrence in cattle due to their skin texture (Jaimes-Dueñez *et al.*, 2018) [10]. According to Maharana *et al.*, 2019 [15], Gujarat has a 40.97% prevalence, most likely due to the ideal warm and humid climate and high tick density that promotes tick survival and piroplasm development which in turn increases the positive rates of babesiosis.

Table 4: Molecular detection of *Babesia* spp. in cattle

Animal	Breed	No. of samples	<i>B. bigemina</i>	<i>B. bovis</i>
Cattle	Kankrej	298	58 (19.46%)	0
	HF cross bred	387	125 (32.30%)	0

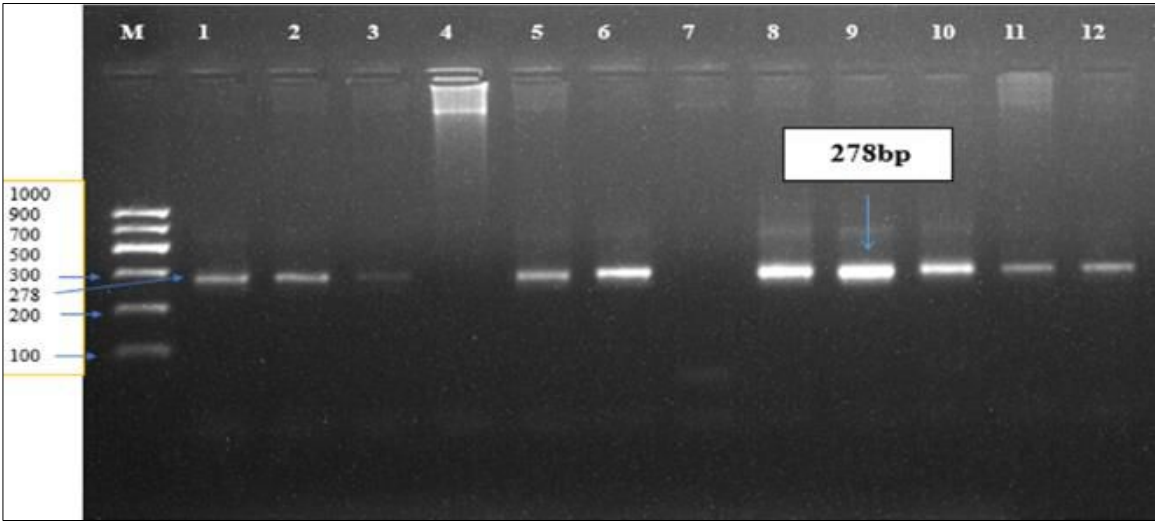


Fig 8: PCR amplicons resolved in 1% agarose gel showing *B. bigemina* positive [M= Marker (100 to 1000 bp), No. 1-3, 5-6, 8-12 (Positive sample of cattle)]

Molecular Characterization of *Babesia* spp. in cattle

Total five purified PCR products of *Babesia bigemina* positive isolates were sequenced using bidirectional sequencing using forward and reverse primers following Sanger’s dideoxy method of sequencing. On quality analysis of these sequences, total 4 sequences were qualified and were used for phylogenetic and homology study using bioinformatic softwares viz. Editseq, Megalign, Bioedit and MEGA 11. Phylogenetic relationship shows the sequences

of the present study are similar with other *Babesia bigemina* sequences reported from the different parts of India. However, they form a different group from other *Babesia* species viz. *Babesia bovis*, *Babesia felis*, *Babesia ovis*, *Babesia divergens*, and *Babesia canis*. Further, all the sequences of the present study form close group and near with Kashmir isolate but away from other sequences from India (UP, Gujarat) and various UK and USA isolates. On homology analysis, it was observed that the sequences of the

present study have 86 to 87 percent similarity among themselves and 80 to 84 percent similarity with other *Babesia bigemina* sequences.

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree is shown in Fig. 5. Evolutionary analyses were conducted in MEGA11

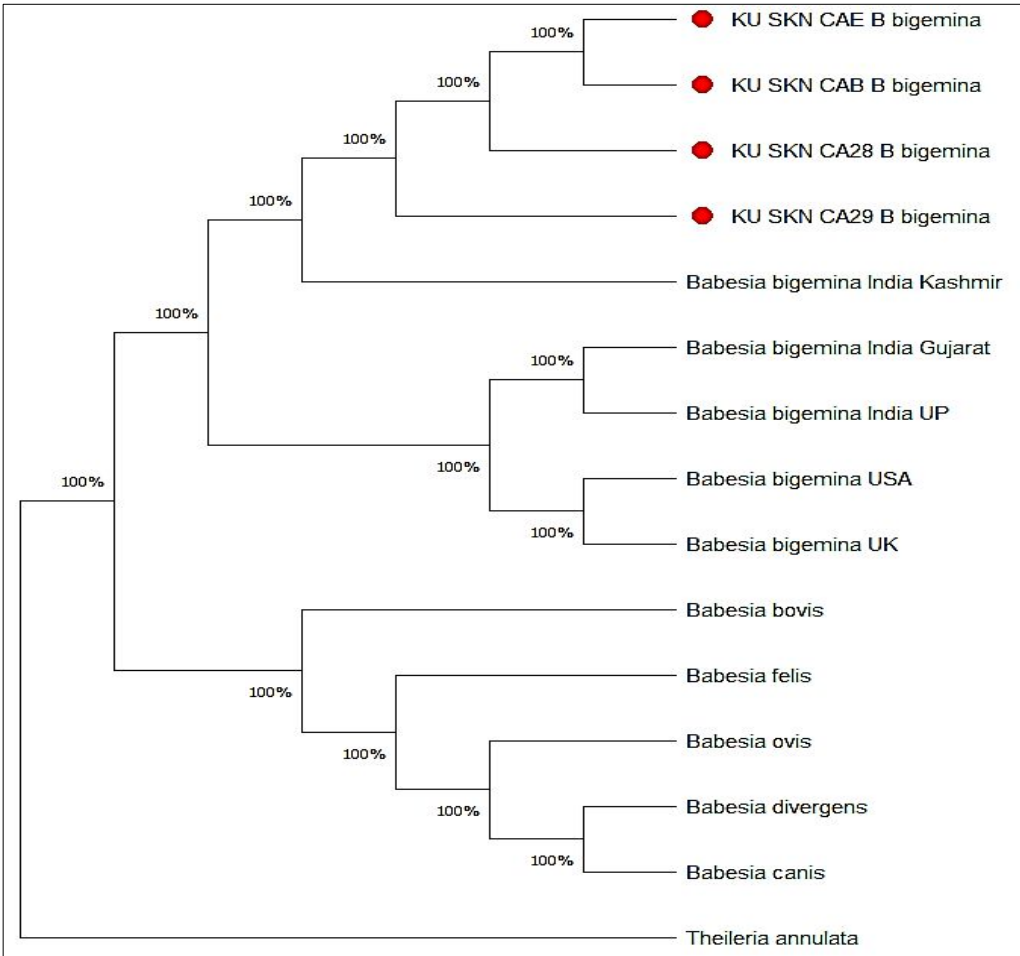


Fig 9: Percent identity and divergence

Percent Identity													
	1	2	3	4	5	6	7	8	9	10	11	12	13
1		86.0	87.9	86.8	84.8	82.3	80.1	82.3	52.2	42.4	55.1	59.3	59.6
2	8.9		90.2	92.7	86.0	81.7	76.7	81.7	49.7	37.6	52.0	58.1	59.0
3	7.5	9.7		92.1	79.2	77.5	74.7	77.5	46.6	36.5	49.7	53.9	54.2
4	8.8	6.8	8.3		83.1	80.3	78.1	80.3	49.7	38.5	51.7	56.7	57.3
5	11.9	2.9	12.3	7.3		93.8	87.6	93.8	61.8	49.4	64.9	71.9	72.8
6	13.3	6.1	12.6	8.9	5.3		86.0	100.0	62.9	49.7	64.6	71.1	71.6
7	20.5	16.7	20.6	15.8	13.0	13.3		86.0	59.0	47.2	61.8	67.4	67.7
8	13.3	6.1	12.6	8.9	5.3	0.0	13.3		62.9	49.7	64.6	71.1	71.6
9	24.0	16.1	24.7	17.7	14.7	15.9	17.1	15.9		78.9	89.9	89.6	89.0
10	45.9	42.3	47.8	41.4	40.6	45.1	42.4	45.1	40.3		81.5	77.2	76.7
11	8.8	2.7	8.8	4.8	0.0	3.7	2.6	3.7	8.3	38.4		92.7	92.1
12	13.5	4.3	13.5	8.2	0.5	4.7	5.2	4.7	14.7	40.6	0.0		99.2
13	13.9	3.8	14.0	8.1	0.0	4.7	5.6	4.7	14.7	40.6	0.0	0.5	
	1	2	3	4	5	6	7	8	9	10	11	12	13

Divergence

KU_SKN_CA28_B_bigemina
KU_SKN_CA29_B_bigemina
KU_SKN_CAB_B_bigemina
KU_SKN_CAE_E_bigemina
Babesia_bigemina_India_Gujarat
Babesia_bigemina_India_Kashmir
Babesia_bigemina_India_UP
Babesia_bigemina_USA
Babesia_ovis
Babesia_divergens
Babesia_bovis
Babesia_felis
Babesia_canis

Fig 10: Phylogenetic relationship

Conclusions

The overall prevalence of *Babesia bigemina* in cattle population of North Gujarat was 127 (18.54%) by microscopy as compared to 183 (26.71%) by PCR technique. Microscopy is a less sensitive and less specific test which needs to be replaced with novel molecular diagnostic tests such as PCR. HF crossbred showed a higher prevalence (24.29% in microscopy and 32.30% in PCR) than indigenous Kankrej cattle breed (11.07% in microscopy and 19.46% in PCR). The prevalence of babesiosis was found to be highest in 1-3 year age group followed by 3-5 and > 5 years and lowest in calves < 1 year old. Summer showed the highest prevalence followed by monsoon and none in winter. Based on phylogenetic analysis, the *Babesia* spp. sequences from present study displayed identity matches with Kashmir sequences.

Acknowledgements

The authors are highly thankful to the Head of Department of Veterinary Parasitology, Principal, College of Veterinary Science and AH, Sardarkrushinagar, and Higher authorities of Kamdhenu University for providing the necessary facilities to carry out this study.

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