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Original Article

The Prevalence and Molecular Characterization of Bovine *Babesia* Species and the First Report of *B. bovis* from Kashmir Himalayas

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Abstract

Background: Bovine babesiosis, a global disease, has not been studied so far in Kashmir valley, which is having temperate type of climate as compared to rest of India having tropical to sub-tropical climate, so we felt the need to investigate it.

Methods: To diagnose the babesiosis in clinically suspected cattle (n=450), peripheral blood film examination and PCR tests using generic and species-specific primers targeting *Babesia/Theleria* genera and *B. bigemina*, *B. bovis* as well as *B. divergens*, respectively were conducted. Four PCR products were sequenced and subjected to BLASTn analysis. Ticks were collected from the clinically suspected animals and identified as per the standard morphological keys.

Results: The prevalence of babesiosis among suspected cattle in central Kashmir by peripheral blood film examination and PCR technique was 11.11% and 33.62%, respectively. The *18S rRNA* gene of Isolate B1 of *Babesia* spp. showed 99.0 to 100% nucleotide sequence homology with *18S rRNA* gene of different isolates of *B. bigemina* registered in the GenBank, while as *18S rRNA* gene of Isolate Z showed 98.5 to 99.2% and 93.1 to 93.9% nucleotide sequence homology with *18S rRNA* gene of different isolates of *Babesia* spp. and *B. bigemina*, respectively, registered in the GenBank. *Rhipicephalus* spp. and *Haemaphysalis* spp. were the two major tick genera identified in the present study.

Conclusion: Bovine Babesiosis in Kashmir is attributed to *B. bovis*, *B. bigemina* and some other *Babesia* spp. or strains which needs further investigation. To our knowledge, this is the first report of *Babesia bovis* from northern India in cattle.

Introduction

Babesia spp. for the first time in India were reported by Walker and Edward in 1927 (1) and among all the *Babesia* spp., *B. bigemina* stands to be the most pathogenic and predominant in India (2) with only few reports of *B. bovis* associated babesiosis in Indian cattle and buffaloes (2,3).

In Kashmir valley, no detailed study has been done with respect to bovine babesiosis except few sporadic cases (4) although occurrence of ovine babesiosis and bovine theileriosis has been established (5, 6). The microscopic detection of *Babesia* parasites in blood smears has always been considered the gold standard for the diagnosis of acute babesiosis, but this detection is difficult in carrier animals with low parasitemia (7). Molecular diagnosis by PCR-based techniques has been developed for diagnosis of such cases and is used for epidemiological surveys in livestock populations (8).

In view of the above background information, the present study was planned to work out the prevalence of babesiosis among suspected cattle in Kashmir with molecular characterization of the prevalent *Babesia* spp.

Materials and Methods

Study area and Animals

The study was conducted in central Kashmir (Srinagar, Budgam and Ganderbal districts) in all the four seasons (winter, spring, summer and autumn) of the year (December 2018 to November 2019). A total of 450 suspected cases of babesiosis presented to various Veterinary Hospitals across central Kashmir were subjected to peripheral blood smear examination and subsequently to PCR for confirmation.

Online survey

To study the occurrence of babesiosis in entire valley i.e., in all the 10 districts (3 in central; 3 in north and 4 in south Kashmir), an online survey was conducted among the field veterinarians by using a mobile application "Surveymonkey" as reported by Zintl, Macgrath & Grady (9) in Ireland. A questionnaire was framed and validated by authors before uploading on "Surveymonkey" web portal (Supplementary file). The link generated was forwarded to 100 field Veterinarians posted at different Veterinary Hospitals of valley regarding babesiosis in cattle.

Peripheral Blood Smear

Thin peripheral blood films (PBF) were examined under oil immersion (1000X) after staining with Giemsa's stain. Thick blood smears were examined for the samples found negative on thin blood films. Morphometric measurement of piroplasms gave some idea for identification of different species of *Babesia.*

Genomic DNA Isolation

Randomly selected 25% blood samples from both positive and negative cases on blood smear examination were subjected to genomic DNA isolation using DNeasy® DNA blood mini kit (Cat No. 51104, Qiagen GmbH, Germany) following the manufacturer's recommendations. The eluted DNA was labelled and stored at -20°C till further use. The concentration (ng/µl) and purity ($OD_{260/280}$) of the extracted DNA samples ranged from 402.9 to 508.9 and 1.71 to 1.86, respectively, using Nanodrop 1000 spectrophotometer (Thermo Scientific, USA).

Polymerase Chain Reaction

All the 25% randomly selected blood samples were subjected to piroplasmid-specific PCR amplification targeting the *18S rRNA* gene of *Babesia* {nearly 400 bp (393-408 bp)} and *Theileria* {more than 400 bp (418-424 bp)} species (Table 1) (10). The randomly selected 75% positive samples on genus-

specific PCR were also then subjected to *B. bigemina*-, *B. bovis*-, and *B. divergens*-specific PCR. The species-specific PCRs target, *SpeI-AvaI* restriction fragment (278 bp) in case of *B. bigemina* (8), *VESA*–1α gene (166 bp) in case of *B. bovis* (11) and *18S rRNA* gene (353 bp) in case of *B. divergens* (12) (Table 1).

Table 1: Primer sequences and Annealing Temperatures

Parasite	Primer sequences (5'-3')	Annealing	References
Piroplasms	F:AATACCCAATCCTGACACAGGG R:TTAAATACGAATGCCCCCAAC	55°C for 1 min.	(33)
B. bigemina	F:CATCTAATTTCTCTCCATACCCCTCC R:CCTCGGCTTCAACTCTGATGCCAAAG	65°C for 1 min.	(8)
B. bovis	F:CAAGCATACAACCAG GTGG R:ACCCCAGGCACATCCAGCTA	50°C for 50 sec.	(11)
B. divergens	F:GTTTCTGACCCATCAGCTTGAC R:CAATATTAACACCACGCAAAAATTC	61°C for 30 sec.	(12)

Sequencing of PCR products and analysis of nucleotide sequences

Four PCR products comprising of two products of *Babesia/Theileria* spp. and one product each of *B. bigemina* & *B. bovis* were purified and sequenced by Xcelris, Ahmadabad, Gujarat, India. The nucleotide sequences were then subjected to BLASTn analysis (13) for determining the similarity with the sequences present in the nucleotide database i.e. NCBI (National Centre for Biotechnology Information). The nucleotide sequences of *18S rRNA* gene, *SpeI-AvaI* restriction fragment and *VESA*–1α gene were edited and analyzed by GENE TOOL and DNA STAR softwares.

Gene sequence data

The nucleotide sequences generated in this study are available in GenBank with accession nos. MT322431; MT322432; MT332223 and MT332222.

Collection and identification of ticks

When present, ixodid ticks were collected from the individual cattle by using blunt forceps. The ticks were processed in the Entomology Laboratory, Division of Parasitology of the University, and identified as per standard protocol (14).

Statistical analysis

The results were subjected to standard statistical analysis reported by Snedecor and Cochran (15). Chi-square test was employed to assess the association of prevalence of babesiosis (calculated on PBF) among cattle with respect to age, sex, breed, and season.

Results

Online survey

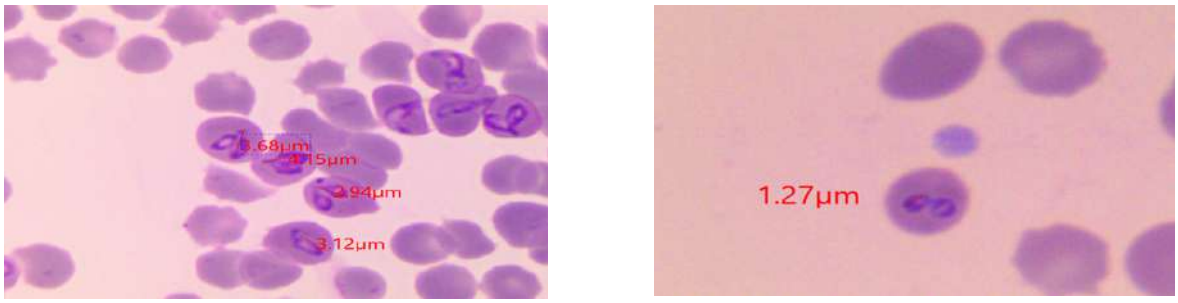
The questionnaire sent to 100 field veterinarians recorded 710 cases. Besides, 300 (42.25%) were reared locally, while 410 (57.74%) were imported from other states of country (Table 2).

Table 2: Online Survey on Bovine Babesiosis

District	*No. of Veterinarians involved in the survey*	*Total no. of babesiosis cases experienced*	*No. of cases diagnosed in bovines brought from other states*	*No. of cases diagnosed in locally reared bovines*
Anantnag	18	119	67	52
Bandipora	3	24	16	8
Baramulla	12	127	95	32
Budgam	15	92	67	25
Ganderbal	12	31	21	10
Kulgam	4	36	32	4
Kupwara	3	6	6	0
Pulwama	11	76	36	40
Shopian	6	83	18	65
Srinagar	16	116	52	64
Grand Total	100	710	410	300

Prevalence of babesiosis

The prevalence of bovine babesiosis on PBF examination taking into account various risk factors is presented in Table 3 and Table 4. Piroplasms detected in the blood films were morphologically diagnosed as *B. bigemina* and *B. bovis*, which was confirmed by PCR based diagnosis and by nucleotide sequences (Fig. 1).



A B

Fig. 1: Giemsa-stained thin blood film (1000X) showing A) *Babesia bigemina* (Isolate LD; large form > 3µm) B) *Babesia bovis* (Isolate DAR; small form <2.5µm)

The prevalence of babesiosis among the suspected cattle using PCR was 33.62% in central Kashmir. Out of 113 (13 PBF positive and 100 PBF negative) samples subjected to PCR for confirmation and detection (latent cases) of babesiosis, only 38 samples amplified using *Babesia* genus specific gene primers with product size of about 394 bp (Fig. 2). Out of these 38 samples, only 29 samples were subjected to PCR for detection of various *Babesia* species. Among them 6 samples amplified using target region specific primers for identification of *B. bigemina* with a product size of 278 bp (Fig. 2), 3 samples showed 166 bp amplification using gene specific primers for identification of *B. bovis* (Fig. 2) and none of the sample showed 353 bp amplification using gene specific primers for identification of *B. divergens*. Thus, on PCR the prevalence of *B. bigemina* and *B. bovis* among the suspected cattle was 20.69% and 10.34%, respectively, and none of the samples showed mixed infection.

Table 3: Prevalence of babesiosis among suspected cattle based on PBF examination

Seasons	*Months*	*Cattle screened*	*No. of +ve cases*	*Age*			*Breed*			*Sex*	
				0-6m	6-24m	>24m	CBHF	CBJ	ND	Male	Female
Autumn	September	60	12	0	0	12	5	7	0	0	12
	October	50	8	0	3	5	2	6	0	2	6
	November	30	1	0	0	1	0	1	0	0	1
	Total	140	21	0	3	18	7	14	0	2	19
	+ve cases		21/140	0	3/48	19/72	7/52	14/84	0/4	2/8	19/132
	%age		15.71%[a]	0%	6.25%	26.38%	13.46%	16.66%	0%	25%	15.15%
Winter	December	30	1	0	0	1	0	1	0	0	1
	January	20	0	0	0	0	0	0	0	0	0
	February	10	0	0	0	0	0	0	0	0	0
	Total	60	1	0	0	1	0	1	0	0	1
	+ve cases		1/60	0	0	1/34	0/21	1/39	0	0	1/60
	%age		1.66%[b]	0%	0%	2.94%	0%	2.56%	0%	0%	1.66%
Spring	March	20	0	0	0	0	0	0	0	0	0
	April	30	1	0	0	1	1	0	0	0	1
	May	40	4	0	0	4	1	3	0	0	4
	Total	90	5	0	0	5	2	3	0	0	5
	+ve cases		5/90	0	0	5/55	2/24	3/59	0/7	0	5/85
	%age		5.55%[a]	0%	0%	9.09%	8.33%	5.08%	0%	0%	5.88%
Summer	June	40	7	2	0	5	1	6	0	1	6
	July	50	8	0	0	7	4	4	0	1	6
	August	70	8	1	0	7	4	4	0	1	7
	Total	160	23	3	0	19	9	14	0	3	19
	+ve cases		22/160	3/24	0	19/103	9/58	14/93	0/9	3/12	19/148
	%age		13.75%[a]	12.5%	0%	18.44%	13.79%	15.05%	0%	25%	12.83%
Total *Babesia* positive cases			50/450	3/58	3/128	44/264	18/155	32/275	0/20	5/25	45/425
			11.11%	5.17%[a]	2.34%[a]	16.66%[b]	11.61%[a]	11.63%[a]	0%	20%[a]	10.58%[a]

+ve =Positive; CBHF= Crossbred local with Holstein Friesian; CBJ= Crossbred local with Jersey; ND= Non-descript.
In last row, for each parameter, the values with different superscripts differ significantly ($P<0.05$).
The values in each positive rows for each season with different superscripts differ significantly ($P<0.05$)

Table 4: District wise % prevalence of babesiosis in cattle on PBF examination

	Srinagar			*Budgam*			*Ganderbal*			*Overall*		
	Imported from other states	Locally reared	Sub total	Imported from other states	Locally reared	Sub total	Imported from other states	Locally reared	Sub total	Imported from other states	Locally reared	Total
Cattle screened	80	95	175	60	90	150	73	52	125	213	237	450
Positive	16	8	24	9	8	17	5	4	9	30	20	50
Incidence (%)	20	8.42	13.71	15	8.88	11.33	6.84	7.69	7.2	14.08	8.43	11.11

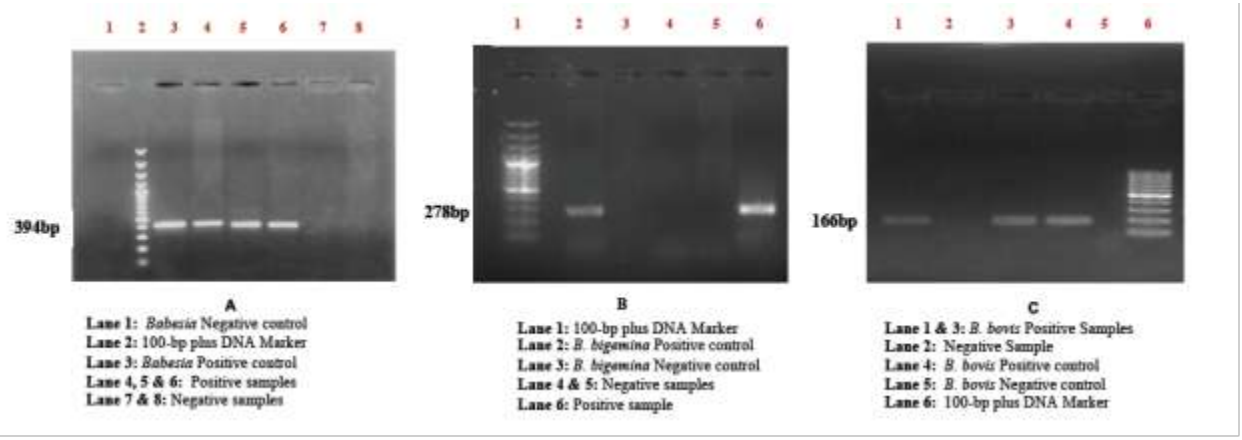


Fig. 2: Amplified A) *Babesia* genus *18S rRNA* gene B) *Babesia bigemina SpeI-AvaI* fragment C) *Babesia bovis VE-SA 1-a* gene

Nucleotide sequence analysis

The total length of the partial *18S rRNA* gene of *Babesia* genus (Isolate B1) was 393 bp (Accession No. MT322431) and of *Babesia* spp. (Isolate Z) was 394 bp (Accession No. MT322432). The nucleotide sequence of Isolate B1 showed 100% similarity with *B. bigemina* Mizoram (MH407694); North East 1 (KF606866) and North East 2 (KF606863); Argentina 1 (HQ688685); Mathura (KY038944) and South Africa (MH257691) Isolates, whereas Isolate Z showed high similarity with *Babesia* spp. Srilanka 1 (LC385886) (99.2%); Bangladesh (MF576177) (98.7%) and Srilanka 2 (LC385888) (98.5%) Isolates. There was a high similarity (99.0% - 99.7%) between Isolate B1 and *B. bigemina* North East 3 (KF606864)/ Argentina 2 (HQ688688)/ Junagadh (MH922769)/ Argentina 3 (HQ688686)/ Argentina 4 (HQ688689)/ North East 4 (KF606865)/ Argentina 5 (HQ688687)/ China 1 (KX115425)/ China 2 (JQ993419) and Vietnam (JN714975) Isolates indicating that the Isolate B1 belongs to *B. bigemina*. The nucleotide sequence homology ranged from 93.1 to 93.9% between Isolate Z and *B. bigemina* Mizoram (MH407694)/ North East 1 (KF606866), North East 2 (KF606863), North East 3 (KF606864) and North East 4 (KF606865)/ Argentina 1 (HQ688685), Argentina 2 (HQ688688), Argentina 3 (HQ688686), Argentina 4 (HQ688689) and Argentina 5 (HQ688687)/ China 1 (KX115425) and China 2 (JQ993419)/ Junagadh (MH922769)/ Mathura (KY038944)/ South Africa (MH257691) and Vietnam (JN714975) Isolates. The nucleotide sequence of Isolate B1 showed 93.4%, 93.6% and 93.4% homology with *Babesia* spp. Srilanka 1 (LC385886), Bangladesh (MF576177) and Srilanka 2 Isolates (LC385888), respectively. The sequence homology between Isolate Z and Isolate B1 was 93.9%. The addition of one nucleotide in Isolate Z compared to Isolate B1 occurred at loci 54, indicating that Isolate Z does not belong to *B. bigemina* (Figs. 3-5). No nucleotide polymorphism was observed for the nucleotide sequence of Isolate B1, whereas two nucleotide polymorphisms were detected in Isolate Z at the loci 232 and 360 of the nucleotide sequence among 20 Isolates in the study.

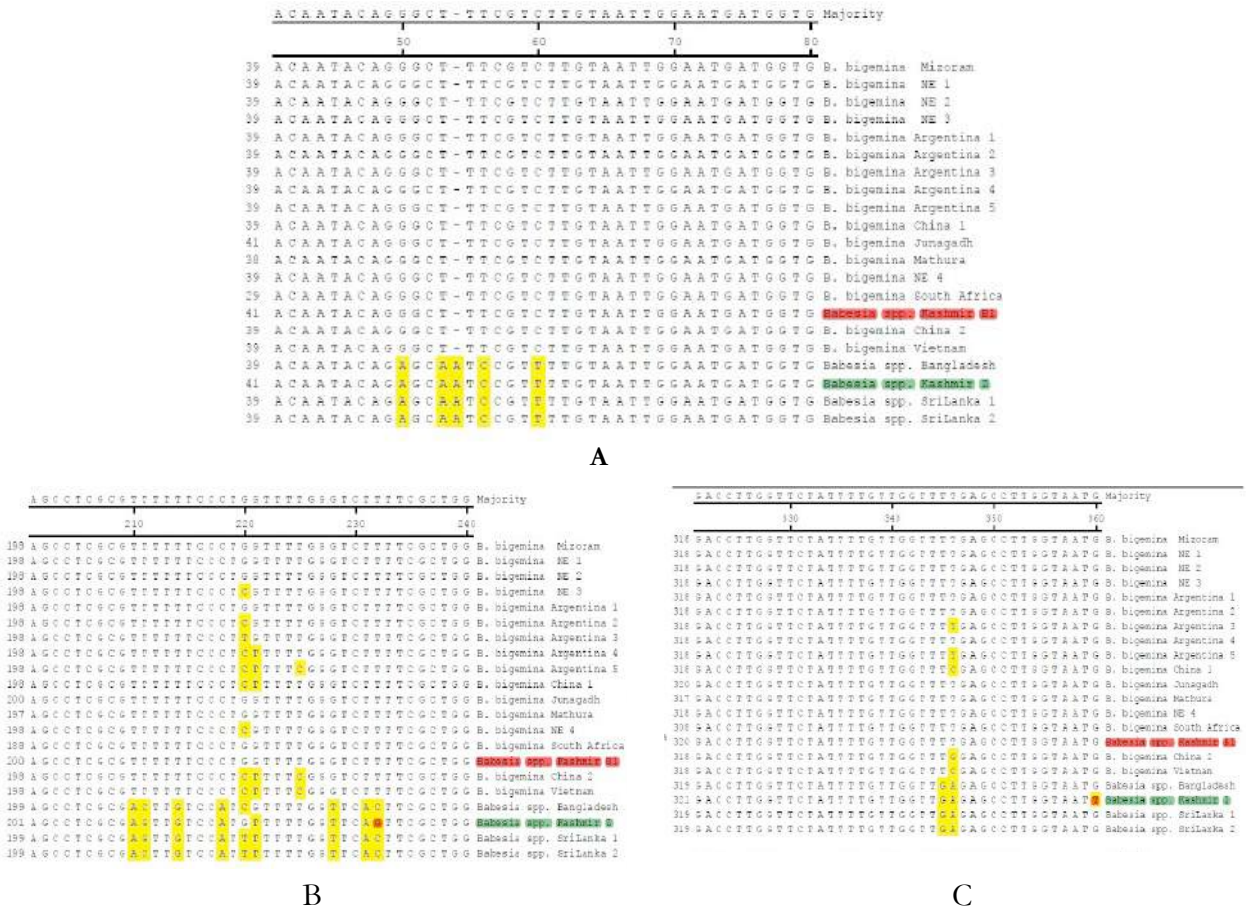


Fig. 3: Prominent alignment results of *18S rRNA* nucleotide sequence of Isolate B1 & Z
Addition of one nucleotide at the loci 54 B) & C) Polymorphism at the loci 232 & 360 in the Isolate Z

The total length of *SpeI-AvaI* restriction fragment of *B. bigemina* (Isolate LD) was 280 bp (Accession No. MT332223). The nucleotide sequence showed only 70.4%, 67.8% and 61.4% similarity with the nucleotide sequence of *SpeI-AvaI* restriction fragment of *B. bigemina* isolated from the different locations of Mexico and USA (S45366), Punjab (AB922127) and Portugal (FJ939724), respectively (Fig. 4 and 5). The total length of the partial *VESA–1α* gene of *B. bovis* (Isolate DAR) was 169 bp (Accession No. MT332222). The nucleotide sequence showed 75.6, 73.1 and 71.9% homology with the nucleotide sequence of *B. bovis* USA P (XM001608260); C2 (XM001611321) and C1 (XM00161107) Isolates, respectively (Fig. 4 and 5).

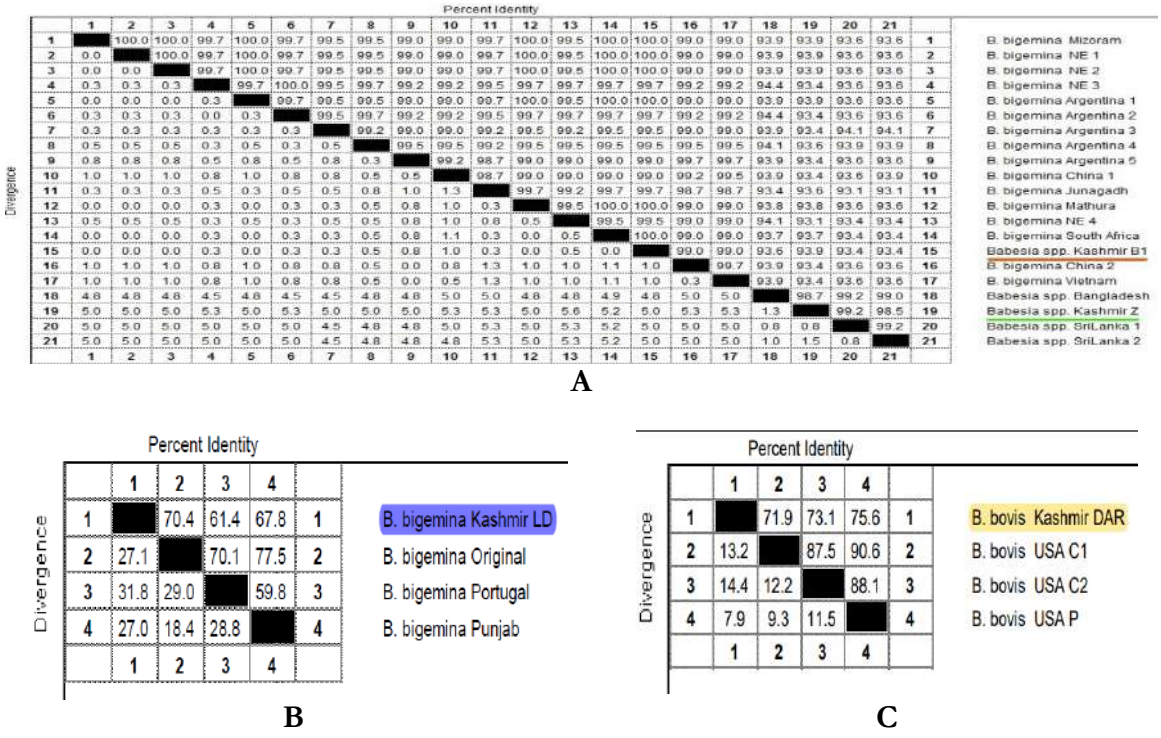


Fig. 4: Pair-wise distances (Nucleotide sequence) of A) *18S rRNA* of *Babesia* Isolates B) *SpeI-AvaI* of B. bigemina C) *VESA-1a* of *B. bovis*

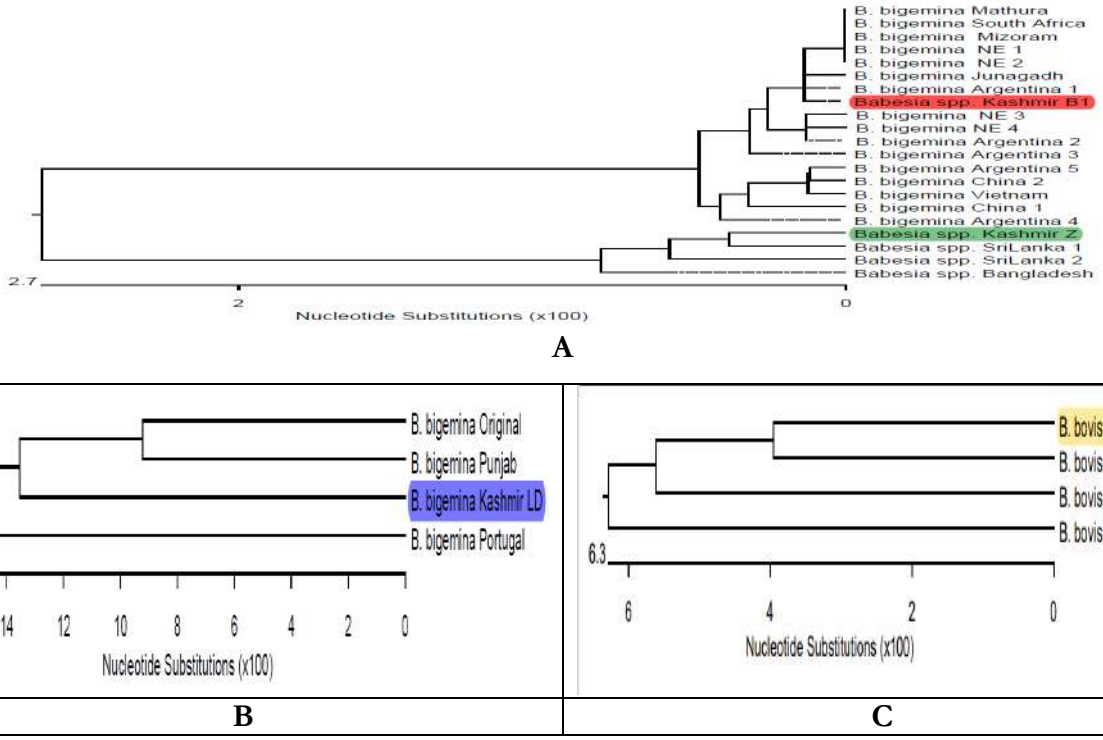


Fig. 5: Phylogenetic tree analysis (Nucleotide sequence) of A) *18S rRNA* of *Babesia* Isolates B) *SpeI-AvaI* of *B. bigemina* C) *VESA-1a* of *B. bovis*

Identification of Ticks

Morphological identification of the collected ticks was done under low power microscope which revealed two major genera of ticks prevalent in the area of investigation *viz., Rhipicephalus* spp. and *Haemaphysalis* spp. *Rhipicephalus* spp. were having short and ridged palpi, hexagonal basis capituli, presence of accessory adanal shields and caudal process in males. *Haemaphysalis* spp. were depicting short and conical palpi, usually sub rectangular basis capituli, trochanter of first pair of legs bear dorsal process.

Discussion

The overall high prevalence of *Babesia* infection in cattle might be due to purposive sampling where cattle showing clinical signs, tick infestation and history of importation from other states of the country were taken into consideration. Rather et al. (16) have observed 7.5% seroprevalence of *B. bigemina* in cattle in District Ganderbal of Kashmir, while as, Shaw (17) and Haq et al. (5) have reported low (1.89%) and high (26.77%) prevalence of *Babesia* infection in cattle and sheep, respectively, in Kashmir. Tick favorable climate in autumn and summer might be the reason for high prevalence in these seasons and corroborates with various workers (18, 19) who reported similar findings. Constable et al. (19) observed high prevalence of haemoprotozoan diseases in rainy season, when vector activity is high.

The age-wise variations in prevalence are in agreement with other observations (20, 21). The low prevalence in young animals in the study could be due to maternal antibodies and/or inverse age-related immunity in young calves up to six months (19). The high prevalence in cattle aged >2 years could be due to pregnancy and lactation stress, and decreased immunity (22). In the present study, the high percentage of babesiosis was recorded in males as compared to females. These findings are in contrast with other observations (16, 23), who reported higher prevalence of bovine babesiosis in females than males. This can be due to the variation in sample size in the present study.

The high prevalence of babesiosis among cattle imported from other states of the country could be due to endemic nature of the disease in those areas (24, 25). The high prevalence of babesiosis in crossbred cattle could be due to low resistance against the haemoprotozoan diseases due to genetic and stress factors during transportation and high milk production, which makes them more susceptible to babesiosis. These stressors augment them to release excess cortisol (6, 7, 22). These findings are in close agreement with the observations of Mahmoud et al. (26) and Rather et al. (16). On the other hand, the animals native to valley may have genetic makeup that makes them resistant to tick infestation as well as *Babesia* infection.

In the present study, the prevalence of babesiosis using PCR technique was higher than the prevalence worked out on PBF examination. This is in line with the fact that the PCR-based assays have the advantage of ensuring the detection of infection in the latent phase of the disease when the level of parasitemia is often below the detection limit of conventional methods (27). Similar findings of higher sensitivity and specificity of PCR over blood smear examination have been reported earlier (28, 29).

In the present study, the nucleotide sequence analysis results of Isolate Z pointed towards the evidence for the existence of a different strain of *B. bigemina* or other *Babesia* spp. This is supported by the fact that two nucleotide polymorphisms were observed at the loci 232 and 360 of the nucleotide sequence of Isolate Z compared to other sequences of *B. bigemina* and *Babesia* spp. in GenBank. Further, Nair (30) also speculated the occurrence of unidentified piroplasms in cattle of northern Kerala and this speculation was supported by Nair et al. (31) who

provided evidence using phylogenetic analysis of *18S rRNA* gene sequences for the existence of *Babesia* spp. other than *B. bigemina* and showed six isolates from Kerala, 98-99%, identical to *B. ovata*. The addition of one nucleotide at loci 54 in Isolate Z compared to Isolate B1 is in line with the nucleotide sequences of *Babesia* spp. registered in the GenBank compared to *B. bigemina* nucleotide sequences in the GenBank.

As *Spe*I-*Ava*I is not a housekeeping gene, therefore, it accounts for low nucleotide homology of the Isolate LD with other sequences of *Spe*I-*Ava*I restriction fragment present in GenBank. Non-amplification of *Spe*I-*Ava*I in Isolate B1 (*B. bigemina*) needs further investigation. The low level of nucleotide similarity of Isolate DAR with the sequences in the GenBank, may be, at least in part, since *VESA*-1 undergoes rapid antigenic variation to evade the host immune response (32) and may be under selective pressure to diversify.

Conclusion

Babesiosis in cattle is endemic in Kashmir valley and is attributed to *B. bovis* and *B. bigemina* and some other *Babesia* spp. or strains which needs further investigation. *Babesia bovis* being one of the species responsible for causing babesiosis in cattle of Kashmir, happens to be the first report from northern India and second report in cattle from entire India. *Rhipicephalus* spp. and *Haemaphysalis* spp. ticks were found to be present on the cattle diagnosed for babesiosis and the same needs further investigation for confirmation of these ticks as vectors of bovine babesiosis in Kashmir valley. *Rhipicephalus* (*Boophilus*) *micropilus* is known to be the primary vector of babesiosis in India and there are reports of transmission of *B. ovata* by *H. longicornis*.

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Conflict of Interest

The authors have declared that no competing interest exists.

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