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Molecular Epidemiology of Bovine Babesiosis in Punjab, Pakistan

Asif Masih, Azhar Rafique, Farhat Jabeen & Shabana Naz

ABSTRACT

***Background*:** Babesiosis is endemic in Pakistan and is one of the most important bovine diseases that causes huge economic losses and high mortality in young animals. This disease is transmitted by a protozoan parasite, which belongs to genus *Babesia* (Apicomplexa: Piroplasmida: Babesiidae). This disease is very much prevalent in summers followed by rainy season because humid environment is favorable for the growth of these parasites. An epidemiological and molecular study was conducted to unveil the prevalence and associated risk factors of *Babesia bigemina* (*B. bigemina*) and *Babesia bovis* (*B. bovis*) in selected districts i.e., Faisalabad, Toba Tek Singh and Jhang of Punjab, Pakistan.

***Materials, Methods & Results*:** A total of 518 (Cattle = 360, Buffalo = 158) blood samples were collected. The samples were analyzed by polymerase chain reaction (PCR) and nested PCR (n-PCR) targeting apocytochrome b-genes (*CYTb*). Chi-square test for univariate analysis was used to analyze the data. The overall prevalence in summer based upon microscopic analysis was 20.55% (37/180) and 13.92% (11/79) in cattle and buffaloes respectively and in winter was 8.80% (16/180), 5.06% (4/79)) in cattle and buffaloes respectively. The samples were further analyzed through conventional PCR (c-PCR) and nested PCR (nPCR). The overall results of conventional PCR in summer showed that 72 cows and buffaloes were infected with babesiosis. The conventional PCR based results of summer showed that prevalence of babesiosis was 29.44% (53/180) in cows and 24.05% (19/79) buffaloes. The results of cPCR during the winter season showed that 12.77% (23/180) and 13.92% (11/79) buffaloes were positive for babesiosis. The overall results of conventional PCR in winter showed that 34/259 cows and buffaloes were infected with babesiosis. On the other hand, the nested PCR results of summer season showed that the prevalence of babesiosis in cows was 32.22% (58/180) and 29.11% (23/79) in buffaloes. In total, 81 cows and buffaloes were infected with babesiosis during summer season. The nPCR results of winter showed that 15% (27/180) cows and 20.25% (16/79) buffaloes were infected with babesiosis. In total, 43 cows and buffaloes were infected with babesiosis. The results have shown that sensitivity of n-PCR is more as compared to conventional PCR. This study is the first molecular evidence of *B. bigemina* and *B. bovis* and its associated risk factors in Punjab province, Pakistan.

***Discussion*:** Dairy sector in Pakistan is one of the fastest growing sectors. Despite of remarkable growth, dairy industry is facing many problems one of them is tick borne diseases (TBDs). TBDs are more prevalent in tropical and subtropical areas of the world and leads to huge economic losses to dairy industry in terms of decreased milk, meat and wool production. Babesiosis is characterized by increased fever, decreased production, poor quality wool, anemia, hemoglobinuria, paleness of mucous membrane. The risk factors analysis of summer and winter data revealed that, adult animals were more prone to babesiosis (24.00%) [$P = 0.032$] and (8.50%) [$P = 0.048$]. In both seasons (summer and winter), females were more infected with babesiosis (20.19% and 8.17%) [$P = 0.049$ and $P = 0.021$] as compared to males, high prevalence in females was might be due to that females were reared for longer period of time. Babesiosis was more occurred in non-cemented floor system (26.01% and 13.51%) [$P = 0.028$ and $P = 0.044$] in summer and winter, respectively. Disease was found more prevalent in closed housing system in summer and winter (27.27% and 10.93%) [$P = 0.043$ and $P = 0.034$] as compared to open housing. Weak animals were more infected with babesiosis (30.84%) [$P = 0.045$] and (12.80%) [$P = 0.042$] in summer and winter, as compared to healthy ones. The animals with high tick infestations were more suffered with babesia infection (25.49% and 13.34%) [$P = 0.036$ and $P = 0.003$] in both seasons as compared to less tick burden.

Keywords: apocytochrome gene, babesiosis, bovine, nPCR, PCR, season.

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Department of Zoology, Government College University Faisalabad (GCUF), Faisalabad, Pakistan. CORRESPONDENCE: A. Rafique [azharrafique96@gmail.com]. Department of Zoology, Government College University Faisalabad (GCUF). Allama Iqbal road. Faisalabad 38000, Punjab, Pakistan.

INTRODUCTION

Bovine babesiosis is one of leading tick-borne infestation facing by dairy industry [25]. This disease is transmitted by a protozoan parasite, which belongs to genus *Babesia* [3,11-13,23]. There are many *Babesia* species exists like *B. bovis*, *B. bigemina* and *B. divergens* which cause the clinical babesiosis in bovines [6,8,10,11,32]. In general, *B. bovis* is considered to be more pathogenic in bovines followed *B. bigemina* and *B. divergens*. Clinical manifestations of disease include high fever, loss of appetite, anemia, hemoglobinuria and paleness of mucous membrane [7,9,15,22,31].

Blood film examinations under microscope is commonly used for diagnosis of *Babesia* but this examination is only used for current and active infection where the parasitemia is high but not useful for carrier animals [2,28,32]. Previously, in Pakistan prevalence has been reported was 2.85%, 2.80% 2.5% and 9.67% in in district Lahore, Peshawar, Kasur and Sahiwal, respectively [33,34].

Very few data based upon the molecular diagnosis of bovine babesiosis is available in Pakistan. The prevalence of babesiosis in district Kasur and southern Punjab in Pakistan was reported 33.33% and 18.75% respectively [11,34]. PCR and Nested PCR (n-PCR) have been found very sensitive particularly in case of bovine babesiosis (*B. bovis* and *B. bigemina*) [7,8,24,27].

The data on diagnosis of babesiosis by two molecular techniques i.e., PCR and n-PCR missing in most parts of the country, keeping in view the fact that the present project was planned to detect the *Babesia* in carrier animals (cows & buffaloes) and to differentiate the *Babesia* species (*B. bovis* and *B. bigemina*) by using specie specific primers.

MATERIALS AND METHODS

Sampling and initial screening

The present study was carried out in the year of 2019 (summer + winter). For this, a total of 518 blood samples from cows (n = 360) and buffaloes (n = 158) were collected in both seasons (summer + winter) from three districts i.e., Faisalabad, Toba Tek Singh and Jhang of Punjab, Pakistan (Figure 1). The samples were randomly collected from healthy animals, initially all the samples were screened via the thin blood smear stained with Giemsa under oil immersion microscope[1] by using all the necessary procedures and protocol [4]. For the molecular investigations, additionally 3-4 mL of blood in vial[2] containing EDTA was also collected for DNA extraction. For studying the associated risk factors a questionnaire was designed and filled in each case.

Molecular investigations of Babesia bovis *and* B. bigemina *DNA extraction*

The genomic DNA from all the collected blood samples (n = 518) were isolated by using raw method i.e., phenol chloroform method by adopting all the protocols [4]. The Genomic DNA of the *Babesia* pathogens isolated from suspected large ruminants - regardless of the ruminant being bovines or buffaloes. The erythrocytes of the animals were first be subjected to a lysis buffer and then proteinase K[3] was introduced to the solution at 10 mg/mL and was allowed for 20 min rest in an incubator at 56°C for around 2 h to allow for the proteinase K[3] for all the erythrocyte proteins in the solution to be digested. Then the extraction agent for isolating DNA, Phenol chloroform isoamyl alcohol[4] was added and the solution was centrifuged at 20000-24000 x *g* for 15 min. The upper phase - clear solution - was taken in a new Eppendorf 2 tube and the bottom layer was discarded. Ethanol 96% was added to make the total volume 3 folds (by adding 2.5X 96% ethanol by volume) and then it was allowed to rest in an incubator for 45 min at -20°C and then it was centrifuged again at 20000-24000 *g* for 15 min. This solution washed in 70% ethanol and the DNA was then be dissolved in TAE buffer[3] (10mM Tris-HCl pH 8, 0.1mM EDTA) at 55°C after it has been air dried. The sample DNA was stored at -20°C for molecular study.

PCR analysis

The DNA samples thus obtained from prospectively infected ruminants (*B. bovis* and *B. bigemina*) was propagated through PCR using the primers[5] (Table 1) for *Babesia* species [23]. These Primers[5] have been designed based on the sequence derived from flanking part of hyper variable region of 18sr RNA. According to the protocol described in the literature; 5 µL of template (sample) DNA + 1X PCR buffer + 0.1 U Taq-Polymerase + 0.5 µL of 20 mM P1/P2 (both primers) + 125 µM of deoxadenosine triphosphate + 125 µM deoxythymidine triphosphate + 125 µM deoxycytidine

triphosphate + 125 µM deoxyguanosine triphosphate) + 1.5 mM $MgCl_2$; totaling 25 µL was placed in PCR tubes[3] and loaded into an automatic thermocycler[6]. The thermocycle scheme used was: 5 min at 95°C (to thermally denature DNA double strands into single strand) then 38 cycles of 45 s at 94°C then 45 s at 56°C then 45 s at 72°C and a final cool down phase of 10 min. The PCR product thus received was then be loaded into an agarose gel block made with 100 mL of 1.5% agarose[3] solution and 3-5 µL of ethidium bromide[4]. The product sizes of *B. bigemina* and *B. bovis* were 394 bp & 260 bp through c-PCR [23] (Figures 4 & 6).

Nested PCR

The samples were further analyzed through the nested PCR (n-PCR) by adopting following protocols found in the literature, the total volume of the PCR mix was 25 µL including 12.5 µL master mix[3], 5.5 µL of ultrapure water, 10 pM of each primer as depicted in (Table 1) and 5 µL of the sample DNA template. Primer annealing temperatures was experimentally derived in a Master Cycler Gradient thermocycler[6]. The PCR protocol - 1 min at 95°C initial denaturation; 35 cycles of 1 min at 95°C then 1 min at 60°C annealing then 1 min at 72°C extension; and a final extension of 5 min at 72°C - n-PCR step was used the same protocol except at step of annealing which was 69°C instead of 60°C. For *B. bigemina* specifically, the PCR conditions were squeezed to be - 2 min at 95°C initial denaturation; 40 cycles of 1 min at 94°C then 30s at 66°C annealing then 1 min at 72°C extension; and a final extension of 5 min at 72°C. The product sizes of *B. bigemina* and *B. bovis* were 250 bp & 195 bp through n-PCR [23] (Figures 3 &5).

Statistical analysis

The obtained data regarding the prevalence and hypothesized associated risk factors was analyzed through the chi-square analysis for univariate by using the Minitab7 19 for windows. Odd ratio was determined to prove degree of association of risk factors. *P* value less than 0.05 was considered significant.

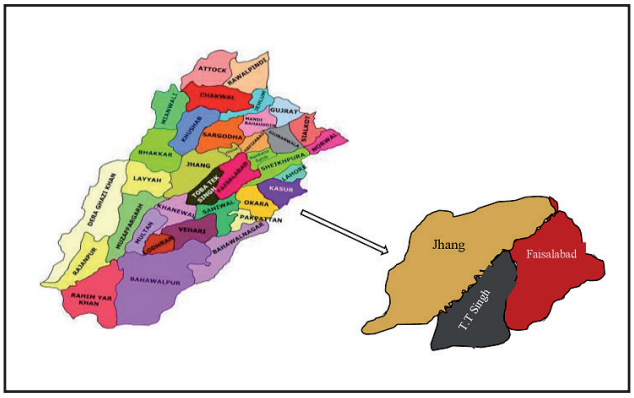

Figure 1. Map of Punjab, Pakistan showing the sampling districts.

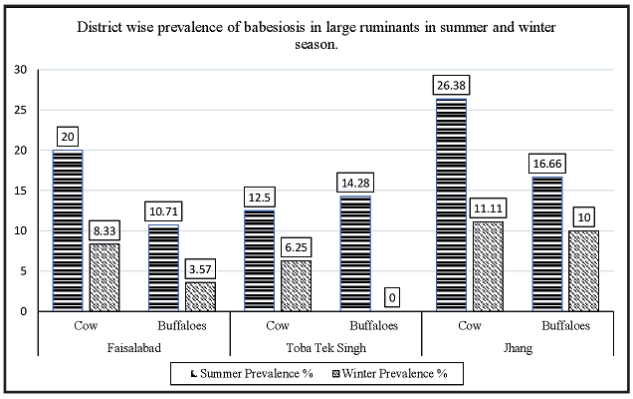

Figure 2. District wise prevalence of babesiosis in large ruminants in summer and winter season.

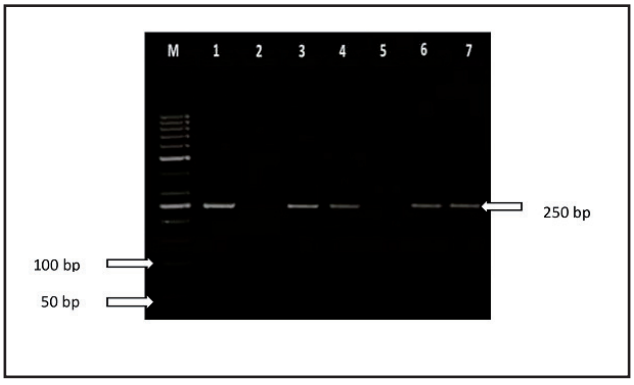

Figure 3. Photograph of sample showing positive results for *Babesia bigemina* (nPCR). M is DNA ladder (50 bp); 1 is control positive; 2 is control negative; samples 3,4 & 6,7 are positive for *Babesia bigemina* while sample 5 is negative.

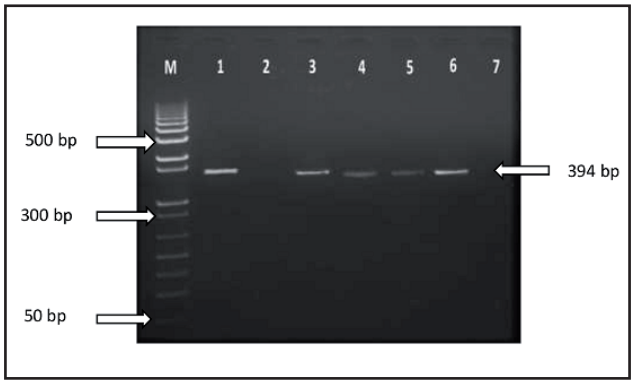

Figure 4. Photograph of sample showing positive results for *Babesia bigemina* (PCR). M is DNA ladder (50 bp); 1 is control positive; 2 is control negative; samples 3,4, 5 & 6 are positive for *Babesia bigemina* while sample 7 is negative.

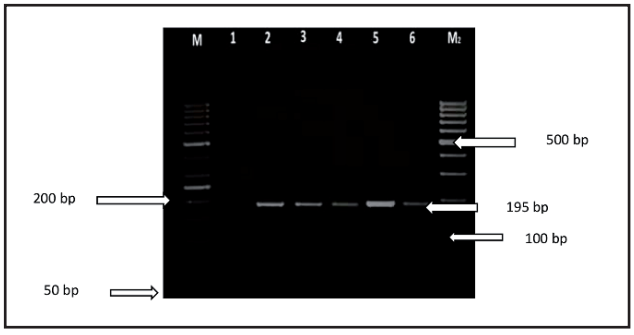

Figure 5. Photograph of sample showing positive results for *Babesia bovis* (nPCR). M is DNA ladder (50 bp); M2 is ladder (100bp); 1 is control negative; 2 is control positive; samples 3,4, 5 & 6 are positive for *Babesia bovis*.

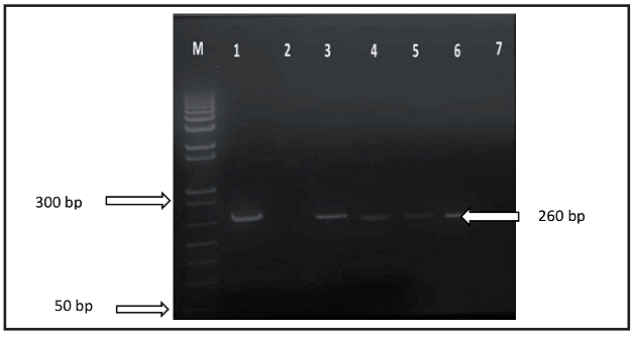

Figure 6. Photograph of sample showing positive results for *Babesia bovis* (PCR).

Table 1. Primer sets used for *Babesia bovis* and *Babesia bigemina.*

Parasite	Assay	Primer	Primer sequence	Size	Reference
B. bovis	PCR	Bbo F Bbo R	TGAACAAAGCAGGTATCATAGG CCAAGGAGATTGTGATAATTCA	260 bp	[23]
	nPCR	iBbo F iBbo R	TCCACGATCTGTGATACGTCA CAAATCCTTTGCAAACTCCAA	195 bp	
B. bigemina	PCR	Bbig F Bbig R	TCCAACACCAAATCCTCCTA CGTGGGTTTCGTTTTTGTAT	394 bp	[23]
	nPCR	iBbig F iBbig R	AAGAGATACCATATCAGGGAACCA TTGGGCACTTCGTTATTTCC	250 bp	

RESULTS

Present study was planned to investigate the molecular detection of babesiosis in large ruminants (cows + buffaloes) in both seasons (Summer + Winter), for this purpose a total of 518 blood samples (n = 518) were collected i.e., cows (n = 360) and buffaloes (n = 158) residing in Faisalabad, Toba Tek Singh and Jhang districts of Punjab, Pakistan. First of all, the samples were examined under microscope by making a thin blood smear. The blood smear exposed parasite schizonts as tear drop in pair identified as babesia. In summer overall prevalence based upon the blood film examinations indicated 20.55 (37/180), 13.92% (11/79) in cows and buffaloes, respectively and in winter was 8.88 (16/180), 5.06% (4/79) in cows and buffaloes, respectively. Furthermore, the prevalence based upon different risk factors i.e., age, sex, feeding, housing, floor system, presence or absence of ticks was determined. Statistical analysis of different risk factors (Table 2) indicated that, increased age, female, non-cemented floor, closed housing, weak body condition and heavy infestation of ticks increase the incidence of disease by many folds.

It was also noted that the district wise prevalence of babesiosis was also different in both seasons i.e., in summer the prevalence in cows was 20.00 (12/60), 12.50 (6/48) and 26.38% (19/72) in Faisalabad, Toba Tek Singh and Jhang, respectively, while in buffalo's prevalence was 10.71 (3/28), 14.28% (3/21) and 16.66% (5/30) in Faisalabad, Toba Tek Singh and Jhang respectively. While in winter the prevalence in cows was 8.33 (5/60), 6.25 (3/48) and 11.11% (8/72) in Faisalabad, Toba Tek Singh and Jhang respectively, while in buffalo prevalence was 3.57 (01/28), 0.00 (0/21) and 10.00% (3/30) in Faisalabad, Toba Tek Singh and Jhang, respectively (Table 3) (Figure 2).

The samples were further analyzed through the conventional PCR (c-PCR) and nested PCR (nPCR). The overall results of conventional PCR in summer showed that 72 cows and buffaloes were infected with babesiosis. The conventional PCR based results of summer showed that 29.44% (53/180) cows and 24.05% (19/79) buffaloes were infected with babesiosis. Among the 53 infected cows 29 were positive for *Babesia bigemina* while the remaining 24 cows were infected with *Babesia bovis*. Similarly, 10 infected buffaloes were positive for *Babesia bigemina* and

9 were infected with *Babesia bovis*. The results of cPCR during the winter season revealed that 12.77% (23/180) cows were found positive for babesiosis. Among these, 16 cows *B. bigemina* was present while in remaining 7 cows *B. bovis* was present. Similarly, 13.92% (11/79) buffaloes were infected with babesiosis. Among them, 7 were positive for *B. bigemina* and 4 were positive for *B. bovis*. The overall results of conventional PCR in winter showed that 34/259 cows and buffaloes were infected with babesiosis (Table 4). On the other hand, the nested PCR results of summer season showed that the prevalence of babesiosis in cows was 32.22% (58/180) and 29.11% (23/79) in buffaloes. Among these infected cows, 34 were *B. bigemina* positive while remaining 24 were positive for *B. bovis*. The 16 buffaloes were positive for *Babesia bigemina* and 7 were positive for *Babesia bovis*. In total, 81 cows and buffaloes were infected with babesiosis during summer season. The nPCR results of winter showed that 15% (27/180) cows and 20.25% (16/79) buffaloes were infected with babesiosis. Among the infected cows, 17 were positive for *B. bigemina* and 10 were positive for *B. bovis*. Similarly, 9 buffaloes were positive for *B. bigemina* while 7 were positive for *B. bovi*s. So, 43 cows and buffaloes were infected with babesiosis in winter (Table 5). The results have shown that sensitivity of n-PCR is more as compared to conventional PCR. The product sizes of *B. bigemina* were 394 bp & 250 bp and of *B. bovis* 195 bp & 260 bp through c-PCR and n-PCR (Figures 3,4,5 & 6).

Table 2. Prevalence of babesiosis in bovine in relation to different risk factors in both seasons (Summer & Winter) based upon microscopy.

Risk factor	Variable	Prevalence %	Chi-square	Odds Ratio	95% CI		*P*-value
					Lower	Upper	
Summer							
Age	Young Adult	12/109 (11.00) 36/150 (24.00)	23.22	2.42	(1.21	2.98)	0.032
Sex	Female male	42/208 (20.19) 6/51 (11.76)	12.33	1.59	(1.31	1.67)	0.049
Feeding system	ground trough	33/148 (22.29) 15/111 (13.51)	14.75	1.96	(1.56	2.32)	0.048
Floor	cemented Non-cemented	16/136 (11.76) 32/123 (26.01)	29.33	2.94	(2.31	3.08)	0.028
Housing	open closed	18/149 (12.08) 30/110 (27.27)	17.43	0.72	(0.41	0.98)	0.043
Herd	>20 animals <20 animals	34/154 (22.07) 14/105 (13.33)	18.44	1.21	(0.43	0.78)	0.048
Body condition	good poor	15/152 (9.86) 33/107 (30.84)	28.44	0.64	(1.01	1.43)	0.045
Ticks	present absent	42/153 (27.45) 06/106 (5.66)	18.94	1.49	(1.16	1.67)	0.036
Dog association	present absent	35/150 (23.33) 13/109 (11.92)	19.42	1.90	(1.34	2.12)	0.033
Winter							
Age	Young Adult	3/72 (4.16) 17/187 (9.09)	19.03	1.59	(1.01	1.22)	0.048
Sex	Female male	17/208 (8.17) 3/51 (5.88)	30.82	2.10	(1.90	2.23)	0.021
Feeding system	ground trough	16/126 (12.69) 4/133 (3.00)	17.43	1.77	(1.42	1.89)	0.043
Floor	cemented Non-cemented	5/148 (3.37) 15/111 (13.51)	21.05	1.40	(1.20	1.56)	0.044
Housing	open closed	6/131 (4.56) 14/128 (10.93)	24.33	2.01	(1.78	2.10)	0.034
Herd	>20 animals <20 animals	14/150 (9.33) 6/109 (5.50)	14.65	0.94	(0.45	1.22)	0.044
Body condition	good poor	4/134 (2.98) 6/125 (12.80)	23.22	1.20	(1.11	1.34)	0.042
Ticks	present absent	18/135 (13.34) 2/124 (1.61)	39.33	2.30	(2.21	2.45)	0.003
Dog association	present absent	14/139 (10.07) 6/120 (5.00)	24.22	1.45	(1.15	1.98)	0.025

$P \leq 0.05$ = Significant; $P \geq 0.05$ = Non- Significant; C.I = 95%.

Table 3. District wise prevalence of babesiosis in small ruminants in summer and winter season based upon microscopy.

District	Variable	Prevalence (%)	Chi- Square	Odds Ratio	95% C. I		*P*-Value
					Lower	Upper	
Summer							
Faisalabad	Cow	12/60(20.00)	13.32	0.78	(0.45	0.97)	0.043
	Buffalo	3/28 (10.71)					
Toba Tek Singh	Cow	6/48 (12.50)	18.95	0.93	(0.35	1.15)	0.049
	Buffalo	3/21 (14.28)					
Jhang	Cow	19/72 (26.38)	21.74	1.49	(1.32	1.54)	0.039
	Buffalo	5/30 (16.66)					
Total	Cow	37/180 (20.55)	20.42	1.78	(1.47	1.90)	0.020
	Buffalo	11/79 (13.92)					
Winter							
Faisalabad	Cow	5/60(8.33)	23.52	1.59	(1.48	1.88)	0.040
	Buffalo	1/29 (3.57)					
Toba Tek Singh	Cow	3/48 (6.25)	19.42	0.84	(0.47	0.90)	0.038
	Buffalo	0/21 (0.00)					
Jhang	Cow	8/72 (11.11)	20.03	1.30	(1.14	1.45)	0.049
	Buffalo	3/30 (10.00)					
Total	Cow	16/180 (8.88)	16.32	0.64	(0.34	0.80)	0.035
	Buffalo	4/79 (5.06)					

$P \leq 0.05$ = Significant; $P \geq 0.05$ = Non- Significant; C.I = 95%.

Table 4. Positive samples for conventional PCR in summer season and winter season.

Species	Total	*Babesia* genus		*B. bigemina*		*B. bovis*	
		Positive	Percentage	Positive	Percentage	Positive	Percentage
Summer Season							
Cow	180	53	29.44	29	16.11	24	13.34
Buffalo	79	19	24.05	10	12.65	09	11.39
Total	259	72	27.79	39	15.05	33	12.74
Winter Season							
Cow	180	23	12.77	16	8.89	07	3.89
Buffalo	79	11	13.92	07	8.86	04	5.06
Total	259	34	13.12	23	8.89	11	4.24

Table 5. Positive samples for nested PCR in summer season and winter season.

Species	Total	*Babesia* genus		*B. bigemina*		*B. bovis*	
		Positive	Percentage	Positive	Percentage	Positive	Percentage
Summer Season							
Cow	180	58	32.22	34	18.89	24	13.34
Buffalo	79	23	29.11	16	20.25	07	8.86
Total	259	81	31.27	50	19.30	31	11.97
Winter Season							
Cow	180	27	15.00	17	9.44	10	5.56
Buffalo	79	16	20.25	09	11.39	07	8.86
Total	259	43	16.60	26	10.03	17	6.56

DISCUSSION

Dairy sector in Pakistan is one of the fastest growing sectors. In national GDP livestock has a shear of about 11.6% along with that 58.9% shear is included in agriculture values in 2017-2018. It is observed that 70% of rural economy is directly or indirectly dependent upon livestock i.e., by selling the milk and meat. Rearing of cattle and buffalo has much importance in rural areas especially for nonagricultural poor families they mostly use horses and donkeys for carrying fodder for their animals [17,26].

Despite of remarkable growth in livestock sector, dairy industry is facing many problems one of them is tick borne diseases (TBDs) [29]. TBDs cause severe losses to animals particularly large ruminants (cattle and buffaloes). TBDs are more prevalent in tropical and subtropical areas of the world and leads to huge economic losses to dairy industry in terms of decreased milk, meat and wool production [14]. Some of diseases are zoonotic in origin and leads to problem in human also. Babesiosis is one of them, which is characterized by increased fever, decreased production, poor quality wool, anemia, hemoglobinuria, paleness of mucous membrane [20,21]. Sometime nervous signs are noted as result of circulation of infected erythrocytes in cerebral blood vessels and birth of weak offspring. Cattle and Buffaloes are more prone to babesiosis and have been reported as endemic in different areas of Pakistan by various studies at different times [16].

Previously this disease was diagnosed microscopically by making a thin blood smear stained with Giemsa and study under the microscope, but this examination is only used for current and active infection where the parasitemia is high but not useful for carrier animals, also it is very difficult to detect *B. bovis* in blood samples. Animals with chronic infection of *Babesia* is difficult to detect because of low number of parasites in blood. Indirect Fluorescent Antibody Test (IFAT) is another serological technique used for the detection of antibodies produced by babesia species (*B. bovis* and *B. bigemina*) due to cross reactivity this assay is also not very much useful to differentiate the species [19]. So, we need assays, which are equally important for both current and active infection as well as for carrier animals.

Therefore, the current study was conducted to examine *Babesia* (*B. bovis* and *B. bigemina*) in naturally infected cattle and buffalos' herds of Faisalabad, Toba Tek Singh and Jhang districts of Punjab, Pakistan through molecular diagnostic techniques i.e., PCR and n-PCR.

In current study, the overall prevalence in summer through microscopic analysis was 20.55% (37/180) in cows and 13.92% (11/79) in buffaloes respectively and in winter was 8.80% (16/180), 5.06% (4/79) in cows and buffaloes respectively. The samples were further analyzed through the conventional PCR (c-PCR) and nested PCR (nPCR). The overall results of conventional PCR in summer showed that 72 cows and buffaloes were infected with babesiosis. The conventional PCR based results of summer showed that prevalence of babesiosis was 29.44% (53/180) in cows and 24.05% (19/79) buffaloes. The 29 cows and 10 buffaloes were positive for *Babesia bigemina* while the remaining 24 cows and 9 buffaloes were infected with *Babesia bovis*. The results of cPCR during the winter season revealed that 12.77% (23/180) and 13.92% (11/79) buffaloes were infected with babesiosis. Among these, 16 cows and 7 buffaloes were infected with *B. bigemina* while in remaining 7 cows and 4 buffaloes *B. bovis* was present. The conventional PCR results of winter showed that 34/259 cows and buffaloes were infected with babesiosis. On the other hand, the nested PCR results of summer season showed that the prevalence of babesiosis in cows was 32.22% (58/180) and 29.11% (23/79) in buffaloes. Among these infected bovines, 34 cows and 16 buffaloes were *B. bigemina* positive while remaining 24 cows and 7 buffaloes were positive for *B. bovis*. In total, 81 cows and buffaloes were infected with babesiosis during summer season. The nPCR results of winter showed that 15% (27/180) cows and 20.25% (16/79) buffaloes were infected with babesiosis. Among the infected animals, 17 cows and 9 buffaloes were positive for *B. bigemina* and 10 cows and 7 buffaloes were positive for *B. bovis*. In total, 43 cows and buffaloes were infected with babesiosis. The results have shown that sensitivity of n-PCR is more as compared to conventional PCR, Similar findings have been reported in southern Punjab, Pakistan [1,34]. According to him 50% samples were positive through molecular techniques i.e., PCR and 3% samples were detected positive via microscopic analysis. Similar results have published and reported that 18% blood samples were positive through PCR as compared to microscopic 3% [23].

Furthermore, the of risk factors analysis of both seasons (summer and winter) revealed that, adult animals were more prone to babesiosis (24.00%) [P = 0.032] in summer and (8.50%) [P = 0.048] in winter as compared to young ones, because the adult animals are active grazers and when they go out for grazing, they are more at risk to ticks secondly immunity decreases with increase in age [18]. Females were more infected with babesiosis in both seasons (20.19% and 8.17%) [P = 0.049 and P =0.021] as compared to males, high prevalence in females was might be due to the fact that females were reared for longer period of time, and female are more prone to physiological stress as compared to male [3]. During this study it was also revealed that non-cemented floor system caused the occurrence of disease in both seasons (26.01% and 13.51%) [P = 0.028 and P = 0.044] as compared to cemented floor system. High occurrence of disease was associated with non-cemented floor system, because in non-cemented floor system there is no proper drainage system, cleanliness of shed along with some cracks in the floor which harbor the parasites that why the eggs of ticks were not properly removed that leads to the incidence of babesiosis in animals [30]. Disease was more prevalent in closed housing system in summer and winter (27.27% and 10.93%) [P = 0.043 and P = 0.034] as compared to open housing this finding was in accordance with this is because of zoonotic nature of disease and disease was more frequent in intensive farming and weak animals were more infected with babesiosis (30.84%) [P = 0.045] in summer and (12.80%) [P = 0.042] in winter, as healthy ones [5]. It was also noted that animals with high tick infestations were more suffered with *Babesia* infection (25.49% and 13.34%) [P = 0.036 and P = 0.003] in both seasons as compared to less tick burden [33]. It was also revealed from present study that feeding system also a major cause of spread of this disease the animals which fed on ground were prone to babesiosis during the summer and winter season (22.29% and 11.11%) [P = 0.048 and P =0.043] [34]. The dog association near herds also caused the transmission of disease in both seasons as ticks were present on dogs (19.42% and 24.22%) [P =0.033 and P = 0.025] and ticks act as an intermediate host [34]. The larger herd size of animals was a major risk factor in the spread of babesiosis (22.07%) [P = 0.043] in summer and (9.33%) [P = 0.044] in winter [11,15].

CONCLUSIONS

Babesiosis is much prevalent in our cattle and buffalos' herds that leads to huge economic losses in terms of poor growth rate, poor quality wool and increased treatment cost after disease outbreak. Commonly smear method is used for detection of blood parasite i.e. *Babesia*, this is not useful for detection of carrier animals or very low parasitemia. Therefore, current study was conducted to investigate the babesiosis in bovine species in Faisalabad Division. PCR for the detection of *Babesia bovis* is specific and sensitive. The test is suitable for tracing carrier animal and provide a qualitative and validated measure that is useful in epidemiological surveys, follow ups for drug treatment and ticks control programs in endemic areas to enhance the livestock productivity.

MANUFACTURERS

[1]Nikon. Tokyo, Japan.

[2]Guangzhou Darui Biotechnology Co. Ltd. Guangzhou, China.

[3]Thermo Fisher Scientific Inc. Waltham, MA, USA.

[4]Sigma Chemical Co. St. Louis, MO, USA.

[5]Macrogen Inc. GeumCheon-Gu, Seoul, South Korea.

[6]BioRad Laboratories Inc. Hercules, CA, USA.

[7]Minitab Inc. State College, PA, USA.

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