

ASSOCIATION OF DIFFERENT RISK FACTORS WITH THE PREVALENCE OF BABESIOSIS IN CATTLE AND BUFFALOS

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This study was planned to conduct a cross-sectional prospective survey in cattle and buffalos of districts Khanewal (southern sandy zone), Faisalabad (central plain zone) and Chakwal (northern arid zone) to determine the epidemiology of bovine babesiosis. A total of 2176 cattle and buffalos randomly selected from each of the study districts were screened through conventional microscopy of Giemsa stained blood films. PCR was performed for 207, 201 and 210 collected blood samples from Khanewal, Faisalabad and Chakwal, respectively. The DNA was amplified using species-specific primers, then subjected to 1.5% agarose gel electrophoresis. Logistic regression and Odds ratio were applied to analyze the data statistically at 95% confidence interval. Babesiosis was found prevalent in the bovine populations of the study districts with lower cumulative distribution of 17.23% (1125/6528) through optical microscopy and higher ($P < 0.05$) of 26.86% (166/618) through PCR. Prevalence was found highest ($P < 0.05$) in district Khanewal (22.75-34.03%) followed in order by districts Faisalabad (18.47-29.85%) and Chakwal (10.48-16.67%). Animal species (cattle-20.19% vs buffalo-14.28%), buffalo breed (Kundi-23.25% vs Nili-Ravi-8.31%), cattle breed (exotic, cross bred and Sahiwal – 29.34%, 21.45% and 10.3%, respectively), sex (female-21.13% vs male-12.68%), age (young-23.17% vs adult-11.9%), animal keeping (tethered-23.48% vs open-11.10%), housing system (closed, semi-closed and open – 22.10%, 19.25% and 9.24%, respectively), hygienic system (very poor, poor and good – 21.04%, 19.78% and 7.07%, respectively), floor pattern (uncemented, partially cemented and cemented – 20.39%, 17.54% and 11.98%, respectively) and season (summer, autumn, spring and winter – 23.41%, 20.47%, 17.77% and 7.29%, respectively) were also found positively associated ($P < 0.05$) with the dissemination of babesiosis in the bovine population of study districts. The study provided baseline data on the distribution of bovine babesiosis in the selected three agro-geoclimatic zones of Punjab, Pakistan which can help in preventive management tools to reduce the risk of disease in the livestock population of Punjab, Pakistan.

Keywords: *Babesia*, Epidemiology, Cattle, Buffalo, Arid, Plain, Sandy.

INTRODUCTION

Bovine babesiosis (vernacular: *Rut Mootra* which means red-colored urine) is a tick-borne (Tb) parasitic disease of ruminants caused by *Babesia* species, a haemoprotozoan parasite including two predominant species i.e. *bigemina* and *bovis*. These species mostly affect cattle and buffalos and are prevalent worldwide (Jaimes-Duenez *et al.*, 2017) with significant distribution in the tropical and subtropical areas of Africa, Asia, Australia, central and south America where tick (Acari: Ixodidae) infestation especially that of *Rhipicephalus* sp. (principal vector for *B. bigemina*) has been reported abundant. Other tick species reported as vectors for babesiosis are: *Boophilus* (*B.*) (*Rhipicephalus*) *annulatus* (Adham and Abd-El-Samie, 2009), *B. decoloratus* (Leefflang and Ilemobade, 1977), *Hyalomma* sp. (Dipeolu and Amoo, 1984) and *Ixodes ricinus* (Reye *et al.*, 2010; Hildebrandt *et al.*, 2010; Nijhof *et al.*, 2010; Ionita *et al.*, 2010; Katargina *et al.*, 2011; Lempereur *et al.*, 2012).

Clinical presentation of the infected animals includes: fever, profound anemia and hemoglobinuria lasting up to 3 weeks (Radostits, 2007; El-Ashker *et al.*, 2015) with modulated haematological and serum profile (Çöl and Uslu, 2006; 2007; Zulfiqar *et al.*, 2012). In severe cases, death may occur within 24 h of the infection (Radostits, 2007). Direct losses of bovine babesiosis such as ill-thrift, abortion, mortality, meat and milk reduction and the cost of control measures can cause serious economic impact on the livestock and dairy industry (Benavides and Sacco, 2007). Field diagnosis of babesiosis can be done through clinical signs and presence of ticks on the animal (Kalume *et al.*, 2009). In heavy infestation, confirmatory diagnosis can also be done through microscopy using Giemsa stain blood smears (Nasir *et al.*, 2000; Alim *et al.*, 2012; Alkareem *et al.*, 2012; Atif *et al.*, 2012b; Fakhar *et al.*, 2012; Jaimes-Duenez *et al.*, 2017). The advent of the DNA-based diagnostic techniques e.g. PCR and RLB have allowed the detection of piroplasms at low parasitemia.

The endemicity of Tb diseases including *Babesia* sp. is higher in Pakistan due to presence of suitable climatic conditions for the development of vectors (Durrani *et al.*, 2010; Rehman *et al.*, 2017; Sajid *et al.*, 2018). The distribution of ticks in different climatic ranges of Pakistan has been documented elsewhere (Sajid *et al.*, 2009; 2011; 2017; 2018; Iqbal *et al.*, 2013; Karim *et al.*, 2017; Rehman *et al.*, 2019). Reports of Tb babesiosis are available from Khyber Paktunkhawa (Iftikhar *et al.*, 2014; Saad *et al.*, 2015; Farooqi *et al.*, 2017), Baluchistan (Kakar, 2013) and Sind (Bhutto *et al.*, 2012). In Punjab, the prevalence of babesiosis in cattle and buffalos has been reported 18.42% in Lahore (Nasir *et al.*, 2000), 29% in Okara (Chaudhry *et al.*, 2010), 6.57% in Rawalpindi, Khushab and Sargodha (Atif *et al.*, 2012a), 25.26% in Chakwal, Faisalabad and Jhang districts (Hassan *et al.*, 2018) and 35.1% tick-DNA pools from semi-arid and arid zones of Punjab, Pakistan (Rehman *et al.*, 2019). A comprehensive update of Tb diseases including babesiosis in Pakistan is reported by Jabbar *et al.* (2015). Under the given situation, arthropod-borne diseases in general and babesiosis in specific are among serious threats to the livestock production in Pakistan which justified the need to investigate the distribution pattern of the disease in various ecologies and husbandry practices of small holder dairy farming system of Pakistan through conventional and/or molecular tools.

MATERIALS AND METHODS

Study Area: The study area comprised of Khanewal (southern sandy zone), Faisalabad (central plain zone) and Chakwal (northern arid zone) districts belonging to different agroclimate of Punjab province, Pakistan. District Khanewal (71°56E, 30°18N) has an area of 3259 Km² with average highest and lowest temperatures per month of 29.8°C and 13.8°C, respectively while average precipitation is 10.5mm. District Faisalabad (73°74E, 30°31.5N) with an area of 1,230 Km² has average highest and lowest temperatures per month of 26.8°C and 16.8°C, respectively while average precipitation is 16.5mm. District Chakwal (72°51 E, 32°55 N) with an area of 6524 Km² has average highest and lowest temperatures per month of 28.63°C and 14.99°C respectively while average precipitation is 42.31mm.

Selection of Animals: Animals were selected from the study districts using simple random sampling with map grids using following formula (Thrusfield, 2018):

$$n = \frac{1.96^2 P_{exp} (1 - P_{exp})}{d^2}$$

Where; n is sample size estimate, P_{exp} is expected prevalence d: absolute precision and 1.96² is a constant for 95% confidence interval.

The sampling frame was designed with farms having at least 10 animals and a distance of 10 Km was kept between the two selected farms.

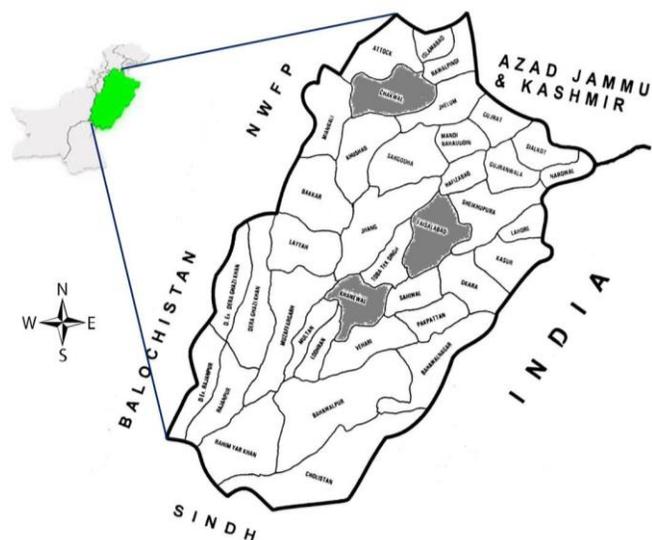


Figure 1. Map of Punjab, Pakistan showing the selected study districts

Collection, Transportation and Examination of Blood Samples: Five mL blood was collected from the jugular vein of 618 animals (207 from district Khanewal, 201 from district Faisalabad and 210 from district Chakwal) and preserved in EDTA-containing vacutainer tubes. The vacutainers were preserved in ice bags and transferred to Molecular Parasitology Laboratory, University of Agriculture, Faisalabad, Pakistan. The blood samples were subjected to form thin blood smear by fixing them with air drying and methanol for 2-3 minutes. Giemsa stain (5%) was used for staining followed by rinsing in the two changes of distilled water buffered to pH 7.2. Examination of the smears was done with compound microscope at 100x by searching at least 50 fields per slide (Adam *et al.*, 1971).

Polymerase Chain Reaction (PCR): The blood samples were subjected to gDNA extraction and amplification using species-specific primers constructed for *B. bigemina* using Primer 3 (version 0.040). The blood samples (200 µl) were added with 400 µl of lysis solution and proteinase K (20 µl). The uniform suspension was made in a 1.5 ml microcentrifuge followed by incubation at 56 °C for 10 minutes. After addition of 200 µl of ethanol (96-100%), lysate was transferred to a Gene JET genomic DNA purification column followed by centrifugation for 1 minute at 6000g. Then washing of lysate was performed in 500 µL wash buffer I (centrifuged for 1 minute at 8000g) and 500 µL of wash buffer II (centrifuged for 3 minutes at 12000g). Finally, DNA was eluted by adding 200 µl of elution buffer to the center of the purification column membrane and incubating it for 2 minutes at room temperature followed by centrifugation for 1 minute at 8000g.

The 18S rRNA was amplified using oligonucleotide primers Bb F 5' GTT CGT TAA CCA CTT TTT CTG GTG 3' and Bb R 5' AGG GAA AAA ACG CGA GGC TG3'. A 25µl PCR reaction tube was used containing 2 mM MgCl₂, 0.2 mM of dnTPs, *Taq* polymerase (0.05 µl), 7µl of DNA, 4µl nuclease free water and set of primers 2 pmol. The PCR was performed in a thermal cycler (C1000 Thermal Cycler, Bio Rad, USA) with one minute initial denaturation at 94 °C followed by 40 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 90s with a final extension step of 72 °C for 10 minutes..

The PCR products (5 µL) were subjected to 1.5% agarose gel electrophoresis carried out with the Power Pac (BioRad, USA) with specific conditions (90 volts and 50 minutes). The gel image was obtained using BioRad Gel documentation system.

Factors Associated with Bovine Babesiosis: Various hosts and their environment-related determinants were considered to determine their association with the prevalence of babesiosis in the study population of the districts. Host-related determinants (animal species, breed, sex, and age) and husbandary practices i.e. animal-keeping (tethered or opened), housing (closed, semi closed or open) hygiene (ranked 1-10 for poor, very poor and good), floor pattern (cemented, partially cemented or non-cemented and seasons of the year were considered to determine an association with the prevalence of babesiosis (if any).

Statistical Analyses: Regression analyses and Odd's ratio (OR) were applied to analyze the data statistically at 95% confidence level (Schork and Remington, 2010). Value of OR > 1.00 described the positive association of determinants with the bovine babesiosis and P-value < 0.05 described significant association of determinants with the dissemination of bovine babesiosis.

RESULTS AND DISCUSSION

Babesia was discovered by Babes (1888) in the cattle population of Romania that caused 50000 mortalities; however, was classified as bacteria at that time. Babesiosis has been reported widely from various regions of the world being lowest (0%) from Iran (Noaman, 2013) and highest

(100%) from Sri Lanka (Jorgensen *et al.*, 1992). Various determinants have been reported for the occurrence of babesiosis e.g. cattle were more at risk than buffalos (Vahora *et al.*, 2012; Li *et al.*, 2014). Exotic and cross-bred were more prone to babesiosis than Sahiwal cattle (Muhanguzi *et al.*, 2010; Atif *et al.*, 2012a), calves were more at risk than adults (Oliveira *et al.*, 2008; Muhanguzi *et al.*, 2010; Jaimes-Duenez *et al.*, 2017) and males were more resistant than females against babesiosis (Alim *et al.*, 2012; Li *et al.*, 2014).

Babesiosis was found prevalent in the bovine populations of all the three districts of the study areas. It was found 22.75% (495/2176), 18.47% (402/2176) and 10.48% (228/2176) in districts Khanewal, Faisalabad and Chakwal, respectively. PCR-based surveillance revealed 34.30% (71/207), 29.85% (60/201) and 16.67% (35/210) distribution in Khanewal (southern sandy zone), Faisalabad (central plain zone) and Chakwal (northern arid zone) districts, respectively. The cumulative distribution was found lower i.e. 17.23% (1125/6528) through optical microscopy than 26.86% (166/618) through PCR (Table 1) which is not different from earlier reports where PCR is more sensitive than microscopy for the detection of *Babesia* sp. in asymptomatic cattle (Galuppi *et al.*, 2012; El-Ashker *et al.*, 2015; Jaimes-Duenez *et al.*, 2017). Hassan *et al.* (2018) revealed that the blood samples of tick-positive animals collected from Chakwal, Faisalabad and Jhang districts were having 25.26% (144/450) positive samples for piroplasms including *T. annulata* and *B. bigemina* amplified using 18S rDNA and RAP-1c genes, respectively through semi-nested PCR. Rehman *et al.* (2019) reported Tb pathogens from Punjab, Pakistan using reverse line blot hybridization assay and found 35.1% (n=142) DNA pools for mixed or single infection of *T. annulata*, *T. orientalis*, *A. marginale*, *A. centrale*, *A. ovis*, *A. platys*-like organism, *B. bigemina*, *B. bovis*, *B. occultans*, *E. minasensis*, *R. massiliae* and two uncharacterized species viz; *Ehrlichia* sp. Multan and *Anaplasma* sp. (BL099-6).

Animal species has also been found statistically associated with the distribution of bovine babesiosis in the study districts. It has been found higher (P<0.05) in cattle than buffalo in study districts which is similar to results of Khan

Table 1. Comparison of diagnostic techniques for bovine babesiosis in three agro-geoclimatic zones of Punjab, Pakistan

District	Levels	Animals Screened	<i>Babesia</i> Positive	Prevalence (%)	Confidence interval 95%		Odds Ratio	P Value
					Lower Limit	Upper Limit		
Khanewal (Southern Sandy Zone)	PCR	207	71	34.30	28.07	40.97	1.51	0.005
	Microscopy	2176	495	22.75	21.02	24.55		
Faisalabad (Central Plain Zone)	PCR	201	60	29.85	23.83	36.45	1.62	0.002
	Microscopy	2176	402	18.47	16.89	20.15		
Chakwal (Northern Arid Zone)	PCR	210	35	16.67	12.08	22.17	1.59	0.017
	Microscopy	2176	228	10.48	09.24	11.82		

et al. (2004). The probable factors contributing towards varied host-susceptibility to babesiosis include: (a) softer skin of the host making it more prone to ticks (Sajid *et al.*, 2009; Kabir *et al.*, 2011; Iqbal *et al.*, 2013) and (b) natural breed resistance of host to ticks (Siddiki *et al.*, 2010). Between buffalo breeds, babesiosis was chronicled higher in Kundi ($P < 0.05$) than Nili Ravi of the study districts; while, among cattle breeds, it was highest in exotic ($P < 0.05$) followed in descending order by cross-bred and Sahiwal breeds. Similar findings have been observed by various scientists in different countries of the world as indigenous cattle has been chronicled resistant from babesiosis and/or ticks than cross-bred (Muhanguzi *et al.*, 2010; Alim *et al.*, 2012; Atif *et al.*, 2012a). Possible causes for the present findings include: (a) enhanced immune response of the native cattle from parasitic diseases due to repeated exposures, (b) lesser exposure of cross-bred cattle due to better management leads to reduced immunity at younger age and higher infections in adult (production) age (Chowdhry *et al.*, 2006; Siddiki *et al.*, 2010).

Animal sex has also been found associated with the distribution of bovine babesiosis being higher in female population ($P < 0.05$) than that in male population of the study zones which is not indifferent from earlier reports (Alim *et al.*, 2012); however, some studies have accordance with our results (Akande *et al.*, 2010; Atif *et al.*, 2012a). Higher dissemination of babesiosis in females might be due to their hormonal disturbances as a result of stress exerted by milk production, breeding (pregnancy, parturition and post-parturition) and drought power that poses them to weakened immune system (Kabir *et al.*, 2011). Youngstock were chronicled with higher abundance ($P < 0.05$) of babesiosis than adults in the study districts. Conflicting reports are available round the globe i.e. some declared calves more susceptible to babesiosis (Muhanguzi *et al.*, 2010) while others reported adults more prone to babesiosis (Khan *et al.*, 2004; Kamani *et al.*, 2010) or not statistically associated with age (Atif *et al.*, 2012a). Possible causes for higher infectivity in youngstock are: (a) lesser or no exposure to the parasites leading to weaker immunity (Kabir *et al.*, 2011) and (b) easily penetration of mouth parts of ticks in the softer skins of young animals (Sajid *et al.*, 2009; Iqbal *et al.*, 2013).

Animal keeping was found associated with the distribution of bovine babesiosis in the study area with higher abundance in tethered animals ($P < 0.05$) than open animals. Possible causes may include: (a) reduced immune response due to tethering stress which leads to increased infection and (b) lesser movement of animals in confinement increasing probability of being attacked by endophilic questing ticks (Sajid *et al.*, 2009; Iqbal *et al.*, 2013). Similarly, the housing system was also positively associated with the dissemination of babesiosis in the bovines of study zones being highest in animals kept in closed housing system ($P < 0.05$) followed in

order by those kept in semi-closed ($P < 0.05$) and opened housing systems. Crevices of bricks and heaps and masses of dung cakes might have higher humidity (due to absence of sunlight) in closed housing providing best sheltering and breeding units for vector population leading to higher disease abundance (Jouda *et al.*, 2004; Muhammad *et al.*, 2008). Due to similar reasons, bovine babesiosis was found highest in animals kept on un-cemented floor pattern ($P < 0.05$) trailed by those kept on partially-cemented and cemented one in the study zones. The poor hygienic system can also be associated with similar reasons for having highest ($P < 0.05$) prevalence.

Highest distribution of babesiosis was found in summer ($P < 0.05$) trailed by autumn ($P < 0.05$), spring ($P < 0.05$) and winter which might be due to favorable environmental condition for growth and development of the vectors (Sayin *et al.*, 2003; Qayyum *et al.*, 2010; Naz *et al.*, 2012).

Association of bovine babesiosis with different determinants in study districts has been summarized in Table 2. Climatographs of bovine babesiosis in districts Khanewal, Faisalabad and Chakwal have been given in Figures 2, 3 and 4, respectively.

It was concluded that *B. bigemina* is the most abundant species bovines of the study districts and PCR improved the diagnosis through detecting false negative results. Risk factors like cattle (species), kundi (buffalo breed) and exotic breeds (cattle), females (sex), calves (age), closed (housing), very poor (hygiene), uncemented (floor pattern), and summer (season) influenced the frequency distribution of bovine babesiosis of the districts. The results of this study provide the baseline information about the spread of disease through climatographs of babesiosis in host population for a calendar year in the study zones of Punjab, Pakistan.

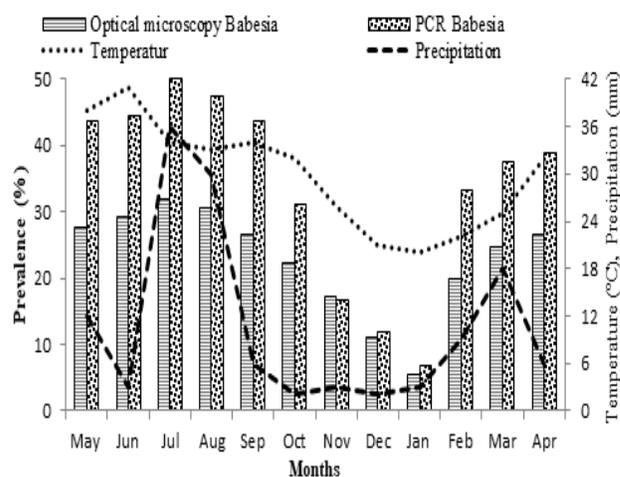


Figure 2. Climatograph of Bovine Babesiosis in District Khanewal, Punjab, Pakistan

Table 2. Associated Risk Factors of Bovine Babesiosis in Three Agro-Geoclimatic Zones of Punjab, Pakistan

Variables	Levels	Animals Screened	Babesia Positive	Prevalence (%)	Confidence interval 95%		Odds Ratio	P Value
					Lower Limit	Upper Limit		
District (Zone)	Khanewal (southern sandy)	2176	495	22.75	21.02	24.55	2.17	0.000
	Faisalabad (central plain)	2176	402	18.47	16.89	20.15	1.76	0.000
	Chakwal (northern arid)	2176	228	10.48	09.24	11.82		
Species	Cattle	3264	659	20.19	18.84	21.59	1.41	0.000
	Buffalo	3264	466	14.28	13.11	15.51		
Buffalo Breed	Kundi	1303	303	23.25	21.02	25.61	2.80	0.000
	Nili Ravi	1961	163	8.31	07.15	09.60		
Cattle Breed	Exotic	961	282	29.34	26.53	32.28	2.85	0.000
	Cross-bred	1254	269	21.45	15.25	23.79	2.08	0.000
	Sahiwal	1049	108	10.30	08.56	12.25		
Sex	Female	3516	743	21.13	19.81	22.51	1.67	0.000
	Male	3012	382	12.68	11.53	13.91		
Age	Young	3090	716	23.17	21.71	24.68	1.95	0.000
	Adult	3438	409	11.90	10.85	13.01		
Animal Keeping	Tethered	3232	759	23.48	22.05	24.97	2.11	0.000
Housing System	Open	3296	366	11.10	10.07	12.21		
	Closed	2063	456	22.10	20.63	24.25	2.39	0.000
Hygienic Measures	Semi- Closed	2561	493	19.25	17.76	20.81	2.08	0.000
	Open	1904	176	9.24	08.00	10.61		
Floor Pattern	Very Poor	2652	558	21.04	19.52	22.62	2.98	0.000
	Poor	2305	456	19.78	18.19	21.45	2.80	0.000
	Good	1571	111	7.07	05.88	08.41		
Season	Un-Cemented	2369	483	20.39	18.80	22.05	1.70	0.000
	Partially Cemented	2582	453	17.54	16.11	19.05	1.46	0.008
	Cemented	1577	189	11.98	10.45	13.66		
Season	Summer	1632	382	23.41	21.40	25.51	3.21	0.000
	Autumn	1632	334	20.47	19.74	23.74	2.81	0.000
	Spring	1632	290	17.77	15.97	19.68	2.44	0.000
	Winter	1632	119	7.29	06.08	08.66		

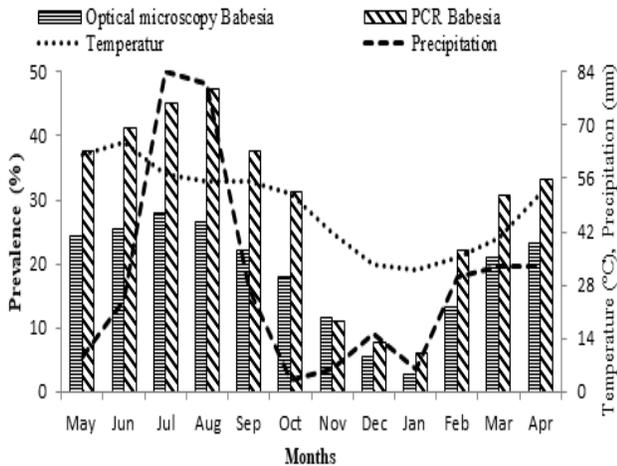


Figure 3. Climatograph of Bovine Babesiosis in District Faisalabad, Punjab, Pakistan

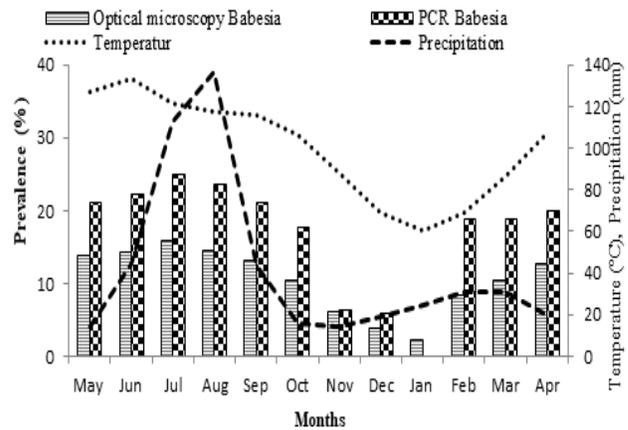


Figure 4. Climatograph of Bovine Babesiosis in District Chakwal, Punjab, Pakistan

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