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ASSOCIATION OF DIFFERENT RISK FACTORS WITH THE PREVALENCE OF BABESIOSISINCATTLE AND BUFFALOS

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This study was planned to conduct a cross-sectional prospective survey in cattle and buffaloes of districts Khanewal (southern sandy zone), Faisalabad (central plain zone) andChakwal (northern arid zone) to determine the epidemiology of bovine babesiosis. A total of 2176 cattle and buffaloes randomly selected from each of the study district 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 and visualized by gel documentation system (BioRad, USA). 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 study districts with lower cummulative 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 babsesiosis 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 parasitic disease of ruminants caused by *Babesia* species. *Babesia* is a haemoprotozoan parasite including two predominant species including *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 ticks species reported as vectors for babesiosis are:*Boophilus (B.) (Rhipicephalus) annulatus* (Adham and Abd-El-Samie, 2009), *B. decoloratus* (Leeflang 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). Haematological and serum profile have been found modulated in animals infected with piroplasmosis (Çöland Uslu, 2006; 2007; Zulfiqar *et al.,* 2012). In severe cases, death may occur within 24 hours 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 such as PCR has allowed the detection of piroplasms at low parasitemia.

The endemicity of *Babesia* species is higher in Pakistan due to presence of suitable climatic conditions for the development of vectors (Durrani *et al.*, 2010; Rehman *et al.*, 2017). The distribution of ticks in different climatic ranges of Pakistan has been documented elsewhere (Sajid *et al.*, 2009; 2011; 2018; Iqbal *et al.*, 2013; Karim *et al.*, 2017).Reports of tick-borne 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) and 6.57% in Rawalpindi, Khushab and Sargodha (Atif *et al.*, 2012a). A comprehensive update of tick-borne 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 is 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. The results of the study might be helpful for planning the control strategies of vector-borne babesiosis not only in selected study districts but, also countrywide.

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 various agroclimatic zones in Punjab province of Pakistan. District Khanewal (longitude 71°56E, latitude 30°18N) has an area of 3259 Km^2 with an average high and low temperatures per month are 29.8°C and 13.8°C respectively while average precipitation is 10.5mm. District Faisalabad (longitude 73°74 E, latitude 30°31.5 N with an area of approximately 1,230 Km^2. Average high and low temperatures per month are 26.8° C and 16.8° C, respectively while average precipitation is 16.5mm. District Chakwal is situated between longitude 72°51 E, latitude 32°55 N with an area of approximately 6524 Km^2. Average high and low temperatures per month are 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 grid method. The sampling frame was designed based on the selection criteria: (a) farms must have at least 10 animals and (b) a distance of 10 Km between the two screened farms. Following formula has been used for calculating sampling size as given by Thrus field (2007):

$$n = \frac{1.96^2 \text{ Pexp } (1 - \text{ Pexp})}{\text{d}^2}$$

Where; n is denoting sample size estimate, P_{exp} showing expected prevalence d: absolute precision and 1.96^2 is a constant for 95% confidence interval.

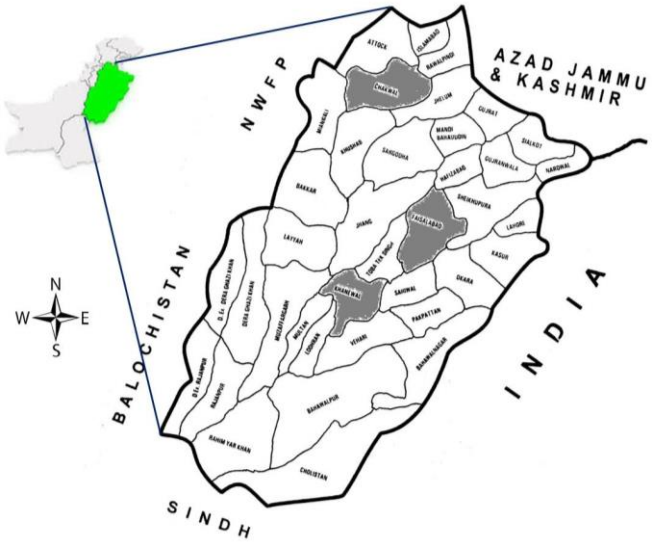

Figure 1. Map of Punjab, Pakistan showing the selected study districts

***Collection, Transportation and Examination of Blood Samples*:** Five mL blood was collected from the jugular vein of the screened animals and preserved in EDTA-containing vacutainer tubes. The vacutainers were preserved in ice bags and transferred to Parasitology Laboratory. 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 100Xby searching at least 50 fields per slide (Adam *et al.*, 1971). A total of six hundred eighteen blood samples (207 from district Khanewal, 201 from district Faisalabad and 210 from district Chakwal) were collected.

***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 $MgCl_2$, 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). 1-minuteinitial denaturation at 94 °C was followed by 40 cycles: a denaturing step of 1 minute at 94 °C, an annealing step of 1 minute at 58 °C, and an extension step of 90 sat 72 °C. The PCR program was ended with a final extension step of 10 minutes at 72 °C.

The PCR products were subjected to 1.5% agarose gel electrophoresis. Five µL sample/PCR amplicon was loaded in the well. The electrophoresis was carried out with the Power Pac (Bio Rad, USA) with specific conditions (90 volts and 50 minutes). The gel image was obtained using Gel documentation system.

***Factors Associated with Bovine Babesiosis*:** Various host 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 included animal species, buffalo breed, cattle breed, sex, and age. Husbandary practices including tethered or opened animals, closed, semi closed or open housing, ranking of hygienic measures from 1-10 and categorization in poor, very poor and good housing, cemented, partially cemented or kachaa floor pattern and seasons of the year were considered to determine the association of climatic factors with prevalence of disease (if any).

***Statistical Analyses*:** Regression analyses and 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 discoverd by Babes (1888) in the cattle population of Romania that caused 50000 mortalities; however, was classified under Bacteria at that time. Babesiosis has been reported widely from various regions of the world. Babesiosis has been reported lowest (0%) from Iran (Noaman, 2013) and highest (100%) from Sri Lanka (Jorgensen *et al.,* 1992). Various determinants have been reported as risk factors for the occurrence of babesiosis e.g. cattle were more at risk than buffaloes (Vahora *et al.,* 2012; Li *et al.,* 2014).Exotic and cross bred were more proned 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, Faisalabad and Chakwal districts, respectively. The cumulative distribution was found lower i.e. 17.23% (1125/6528) through optical microscopy than 26.86% (166/618) through Polymerase chain reaction (PCR) (Table 1) which is not different from earlier reports where PCR is more sensitive than microscopy for the detection of *Babesia* spp. in asymptomatic cattle (Galuppi *et al.,* 2012; El-Ashker *et al.,* 2015; Jaimes-Duenez *et al.,* 2017). Prevalence was found significantly highest (P<0.05) in district Khanewal (southern sandy zone) followed in order by district Faisalabad (central plain zone) and district Chakwal (northern arid zone).

Animal species has also been found statistically associated with the distribution of bovine babesiosis in the study districts. Ithas been found higher (P<0.05) in cattle than buffalo in study districts. The probable factors contributing towards varied host-susceptibility to babesiosis include: (a)

Table 1. Comparison of diagnostic techniques for *Bovine babesiosis* in three Agro-geoclimatic zones of Punjab, Pakistan

District	Levels	Animals Screened	*Babesia* 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
	Optical 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
	Optical 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
	Optical Microscopy	2176	228	10.48	09.24	11.82		

softer skin of the host making it more prone to tick vector (Sajid *et al*., 2009; Kabir *et al*., 2011; Iqbal *et al*., 2013) and (b) natural breed resistance of host to tick vectors (Siddiki *et al*., 2010). Between buffalo breeds, the distribution of bovine babesiosis was chronicled higher in Kundi (P<0.05) than Nili Ravi of study area. Among cattle breeds, prevalence of babesiosis has been found highest in exotic breed (P<0.05) followed in descending order by cross-bred cattle (P<0.05) and Sahiwal breed. Similar findings have been observed by various scientists in different countries of the world as indigenous cattle has been chronicled resistant from babesiosis than cross bred (Muhanguzi *et al.,* 2010; Alim *et al.,* 2012; Atif *et al.,* 2012a). Possible causes for the present findings include: (a) enhanced immue response of the native cattle from parasitic diseases due to repeated exposures, (b) lesser exposoure of cross-bred cattle from parasitic infection due to its better management leads to reduced immunity at younger age and higher infections in adult (production) age (Chowdhury *et al.,* 2006; Siddiki *et al*., 2010).

Animal sex has also been found statistically associated with the distribution of bovine where prevalence of babesiosis has been found higher in female population (P<0.05) than that of male population of study zones. The result of current research is not indifferent from earlier reports (Alim *et al.,* 2012) however the findings of some studies are having in accordance with the present study (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-partirition) and drought power that poses them to weakened immune system (Kabir, *et al.,* 2011).Young animals (P<0.05) have been chronicled with higher abundance of bovine babesiosis than adults in study districts. With respect to age-wise comparison, conflicting reports are available round the globe. Some reports declared calves more susceptible to babesiosis (Muhanguzi *et al*., 2010) while others reported adults more proned than calves (Kamani *et al*., 2010; Atif *et al*., 2012a). Possible causes for the more disease occurrence in young animals are: (a) lesser or no exposure to the parasites at young age leads to lesser immune response (Kabir, *et al.,* 2011), (b) mouth parts of tick vector can easily penetrate in the soft skin of young animals (Sajid *et al*., 2009; Iqbal *et al*., 2013).

Animal keeping has also been found statistically associated with the distribution of bovine babesiosis in the study area where its prevalence is found higher in tethered animals (P<0.05) than open animals. Possible causes may include: (a) reduced immune response due to rope stress which leads to increased parasitic infection, (b) probability of being attacked by the vector will be higher in tethered animals due to lesser movement of animals in confinement (Sajid *et al.,* 2009; Iqbal *et al.,* 2013).Similarly, the housing system has also been recorded positively associated with the dissemination of babesiosis in the bovine population where its prevalence is found highest in animals kept in closed housing system (P<0.05) followed in order by those kept in semi-closed housing system (P<0.05) and opened housing system. Possible causes may include: (a) heaps of bricks and masses of dung cakes having higher humidity (due to absence of sunlight) in closed housing system are the best breeding units for vector population and leads to higher disease outbreak (Muhammad *et al.,* 2008) (b) the best sheltering places for vector ticks are closed housing system (Jouda *et al.,* 2004). Due to similar reasons, bovine babesiosis have also been found association with the floor pattern of farms being highest in animals kept on un-cemented floor pattern (P<0.05) trailed by those kept on partially-cemented and cemented floor pattern in study zones. The poor hygienic system has also been recorded having similar probability of highest (P<0.05) prevalence probably due to optimum breeding microclimate for the vectors.

Season has also chronicled positively associated with the distribution of babesiosis in the bovine population of study area where prevalence has been found highest in summer season (P<0.05) trailed by autumn season (P<0.05) than spring season (P<0.05) and lowest in winter season. Summer season has also been chronicled with highest disease existence in various regions of the world 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. Various risk factors have been reported to be statistically associated with the epizootiology of babesiosis in ruminant population of Pakistan in earlier studies e.g. calves found more proned to babesiosis than adults, male animals chronicled resistant to bovine babesiosis than females and exotic and cross bred cattle have been reported with higher abundance of babesiosis than indigenous Sahiwal cattle (Atif *et al.,* 2012a). Prevalence of haemo-parasites has been reported higher in cattle population than buffaloes and adult population than young animals (Khan *et al.,* 2004). The prevalence of theileriosis in bovines has been reported higher in calves, females and exotic breeds as compared to adult, males and local breeds respectively and in summer season as compared to other seasons (Muhammad *et al.,* 1999; Qayyum *et al.,* 2010Durrani *et al.,* 2010; Atif *et al.,* 2012a; Naz *et al.,* 2012).

It is concluded that babesiosis (*Babesia bigemina* is the prevalent species) is prevalent in cattle and buffalos in decreasing order of abundance in district Khanewal, Faisalabad and Chakwal representative of all the three agro-geo climatic zones of Punjab, Pakistan. PCR improved the diagnosis through detecting false negative results.

Table 2. Associated Risk Factors of Bovine Babesiosis inThree 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		
BuffaloBreed	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 Breed	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 Cattle	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	Tethered	3232	759	23.48	22.05	24.97	2.11	0.000
Keeping	Open	3296	366	11.10	10.07	12.21		
Housing	Closed	2063	456	22.10	20.63	24.25	2.39	0.000
System	Semi- Closed	2561	493	19.25	17.76	20.81	2.08	0.000
	Open	1904	176	9.24	08.00	10.61		
Hygienic	Very Poor	2652	558	21.04	19.52	22.62	2.98	0.000
Measures	Poor	2305	456	19.78	18.19	21.45	2.80	0.000
	Good	1571	111	7.07	05.88	08.41		
Floor Pattern	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		

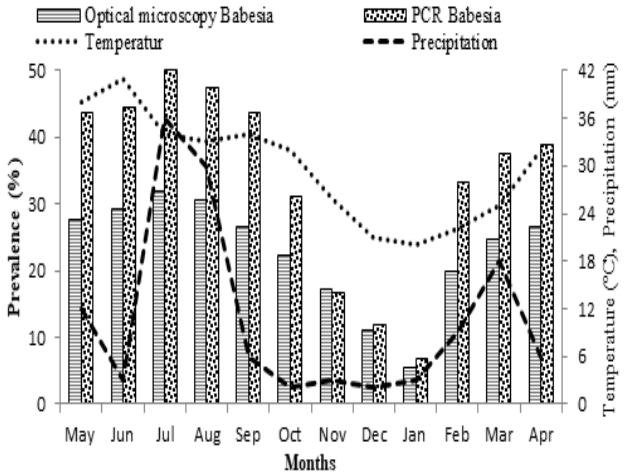

Figure 2. Climatograph of Bovine Babesiosis in District Khanewal, Punjab, Pakistan

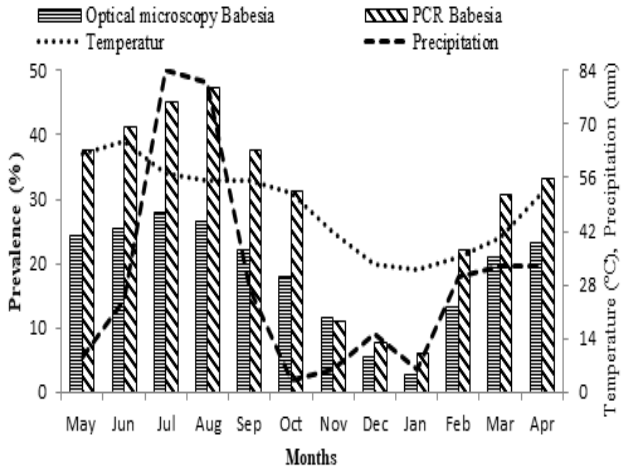

Figure 3. Climatograph of Bovine Babesiosis in District Faisalabad, Punjab, Pakistan

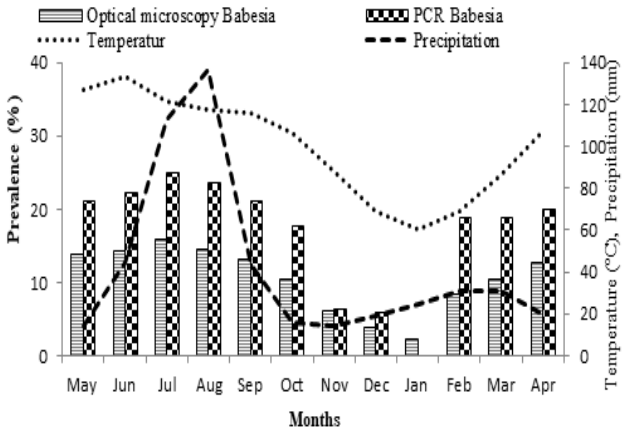

Figure 4. Climatograph of Bovine Babesiosis in District Chakwal, Punjab, Pakistan

Risk factors like cattle (species), kundi (buffalo breed) and exotic breeds (cattle), females (sex), calves (age), closed (housing system), very poor (hygiene), uncemented (floor pattern), and summer (season) influenced the frequency distribution of bovine babesiosis of the districts. There is a need of planning strategies to control the vector-borne diseases in the study districts throughout the year generally and during an outbreak season especially. 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. Other relevant information on the epizootiology of babesiosis and risk factors statistically associated with babesiosis may help an integrated control of the manance from the indigenious farming system of Pakistan.

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