Epidemiology and Molecular Detection of Babesiosis in Household Dairies in Districts Kohat and Karak, Khyber Pakhtunkhwa Pakistan

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Abstract: Back ground; Babesiosis is economically important disease which causing huge mortality and morbidity to the livestock sector particularly in the under developed countries including Pakistan. **Result;** A total of 2400 Cattle blood samples (1200 Calves and 1200 Cows) were examined through PCR and microscopy in karak and kohat districts of Khyber Pakhtunkhwa. The overall prevalence of Babesiosis in cattle was found 9.875% (237/2400) by microscopic technique which were followed by district Karak 9.25 % (111/1200) and district Kohat 10.5 % (126/1200).The highest seasonal prevalence of Babesiosis was recorded in the summer 20.375% (163/800) followed by spring 9% (36/400), autumn 7.25% (29/400) and the lowest was seen in the winter 1.125% (9/800). In the present study, female cattle showed high prevalence 11.22% (184/1639) rate of Babesiosis as compare to male cattle 6.96% (53/761). The PCR based Prevalence rate of Babesiosis was found 27.5% (165/600) among this 24% (72/300) prevalence rate was noted in district Karak and 31% (93/300) in district Kohat. Babesia bovis 541bp and Babesia bigemina 1124bp amplified DNAs were visualized through gel electrophoresis. **Conclusion;** It is revealed from the present study that PCR was more sensitive than microscopy in diagnosis of the Babesiosis in House hold dairy cattle
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Key words: PCR, Microscopy, Babesiosis and house hold dairy

1. Introduction

Babesiosis is an important andfatal disease of cattle caused by protozoan parasites of the genus *Babesia*. It is an economically important disease which causing huge mortality and morbidity to the livestock sector particularly in the under developed countries including Pakistan. The disease is transmitted through ticks in cattle, buffaloes, sheep and goats world widely, especially in the tropical and subtropical regions. Babesiosis is the second most common blood-borne parasitic disease of mammals after the trypanosome [1, 2, 3, 4].More than 100 species of *Babesia* have been recognized morphologically which infect wide groups of animals. *Babesia bovis* and *Babesia bigemin*a are more important and vastly predominant in tropical and subtropical countries causes huge losses to the livestock industry [5, 6, 7]. Babesiosis causes about 90% deaths in cattle when they were not provided health facilities [8, 9] while it was reported that Babesiosis causes 30% deaths in cows and 70-80% in sheep [10]. In several countries of the world including Asia, Australia, Africa, South and Central America and United States the occurrence of Babesiosis in cattle is about 1.2 billion [11, 12] while Niazi*et al* (2008) reported 5.5- 42.8% prevalence of *Babesia* infection in cattle and buffaloes in Pakistan [13]. Babesiosis has a great monetary impact due to mortality, loss of meat, beef and milk productions of infected animals as well as this disease also have great influence on international dairy trade [5, 14]. It is essential to detect the disease to control and prevents the spreading of Babesiosis. *Babesia spps* can be detected easily through microscopic technique in the red blood cells of the host at the acute stage of Babesiosis. Polymerase chain reaction (PCR), which is more sensitive and specific technique, offers an alternative approach for the diagnosis of Babesiosis [12] in every age group of animals.Keeping in view, the importance of the disease due to economic losses to the dairy industries the research work was designed to study the epidemiology of Babesiosis in house hold dairies in district Karak and district Kohat of Khyber Pakhtunkhwa.

2. Materials and Methods

2.1. Sources and collection of specimen

A total of 2400 blood samples were collected randomly from the house hold dairy of different locations in district Karak and Kohat during the period January to December 2012. The blood were collected directly by syringe from the jugular vein in sterilized vacationer having capacity of 5ml each then labeled with date, sex , area and nature of animal. The samples were placed in ice jar and transported to

laboratory of molecular Parasitology and virology Kust kohat for further process.

2.2. Slides preparation

Thick and thin blood smears were prepared and fixed with absolute methanol for one minute. Fixed slides were stained in working dilution of Giemsa's stain (1:10) for 30 min. The slides were washed with tap water and dried in air. Stained slides were observed under 100x magnification using microscope (Olympus Japan) to detect *B. bigemina* and *B. bovis* .Morphological characteristics of *Babesia species* were identified according to key described by [15]

2.3. DNA extraction.

A Total of 600 blood samples (300 from each district) DNA were extracted through Tissue DNA extraction kit (Vivintis, USA) as per followed the manufacturer protocol.

2.4. Amplification of DNA (Polymerase chain reaction)

PCR technique was used for the amplification of *B. bovis* and *B. bigemina* DNA using the primer sequences for B.bovis forward GAU9 (5-CTGTCGTACCGTTGGTTGAC-3) with target position of the genome is 675-694 bp and the reverse primer GAU10 (5-CGCACGGACGGACGGAGACCGA-3) with the target position1215-1198.and similarly for Babesia bigemina the forward primer GAU5 (5-TGGCGGCGTTTATTAGTTCG-3) and reverse primer GAU6 (5-CCACGCTTGAAGCACAGGA-3).The reaction mixture was containing 10xPCR buffer 2.0ul,$Mgcl_2$ (25mM) 2.5ul,dNTPs(10mM) 1.0ul forward primer(P1) 1.5ul,Reverse primer (p2) 1.5ul, Tag DNA polymerase (2u/ul) 1.0ul and extracted DNA 2.0 ul and separate reaction mixture for B.bigemina primer (P3 and P4) .The cycling condition for regular PCR was initial denaturation temperature 94^0c for 10minute, annealing temperature of 35cycle with 94^0c for 30sec,50^0c for 30sec, 72^0c for 45sec and extension 720c for 10 minutes. [17]

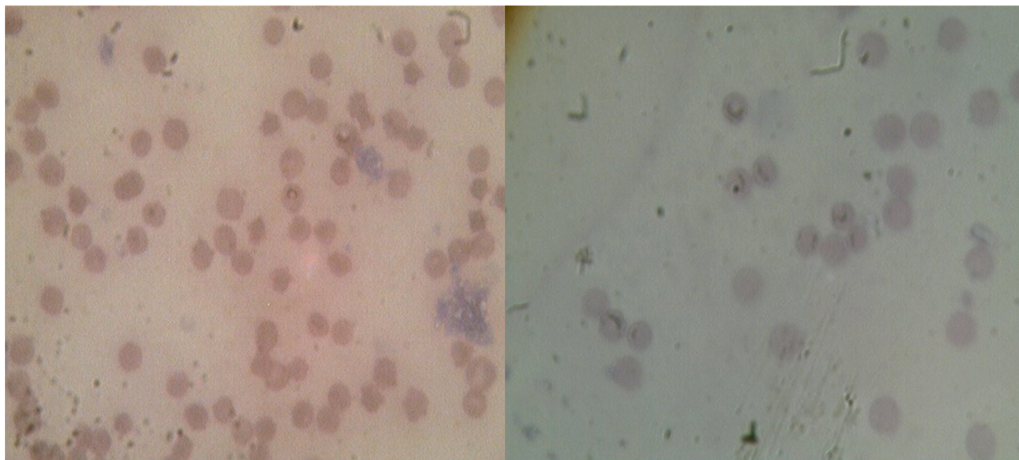
2.5. Analyses of the PCR product.

The PCR products were analyzed in 2g Agarose gel with ethidium bromide (1µg/ml) stain and compare with 50bp DNA ladder . The gel was then placed on transilluminator and observed 54bp products of *B.bovis* and 1,124 bp products of *B.bigemina*

3.0. Results and Discussion

The overall prevalence of Babesiosis in two districts, karak and kohat Khyber Pakhtunkhwa was 9.875% (237/2400). Among these, in District Karak 9.25% (111/1200) and in district Kohat 10.5 % (126/1200) were found by microscopy during the study (Table.1 and Fig 1) The high prevalence of Babesiosis was found in the month of July 30.87% (61/200), followed by June 20.50% (41/200), August 17% (34/200) and the lowest was noted in November 2% (4/200). However there was no positive / *Babesia* infected Cattle reported in months of January and December 2012 (Table.1). The high prevalence of Babesiosis was found in the summer 20.375% (163/800) followed by spring 9% (36/400), autumn 7.25% (29/400) and the lowest was seen in the winter 1.125% (9/800) (Table.2)

The high prevalence of Babesiosis was noted at the age of 4 years and above 13.4% (61/452) followed by 11.7% (48/409) at the age of 3-4 years while the lowest was found at the age of 0-6 months .In the present study, female cattle showed high prevalence 11.22% (184/1639) rate of Babesiosis as compare to male cattle 6.96% (53/761)(Fig.2).



Fig; 1. Showing the Babesia bovis and Babesia bigemina amastigote in the RBC of the Cattle.

Table.1 Prevalence of Babesiosis in Cattle of district Karak and Kohat KPK, Pakistan

Months	Karak			Kohat			Total % (Total)
	Cow +	**Calves+**	**+ Total**	**Cow +**	**Calves+**	**+ Total**	
January	0	0	0/100	0	0	0/100	0%(0/200)
February	1	0	1/100	2	2	4/100	2.5%(5/200)
March	3	2	5/100	7	4	11/100	8%(16/200)
April	5	2	7/100	8	5	13/100	10%(20/200)
May	7	4	11/100	9	7	16/100	13.5%(27/200)
June	11	7	18/100	14	9	23/100	20.5%(41/200)
July	17	15	32/100	18	11	29/100	30.5%(61/200)
August	12	6	18/100	10	6	16/100	17%(34/200)
September	7	5	12/100	6	3	9/100	10.5%(21/200)
October	3	2	5/100	2	1	3/100	4%(8/200)
November	1	1	2/100	1	1	2/100	2%(4/200)
December	0	0	0/100	0	0	0/100	0%(0/200)
Grand Total	67/600	44/600	**111/1200 9.25%**	77/600	49/600	**126/1200 10.5%**	**9.875%(237/2400)**

Table.2. Season wise Prevalence of Babesiosis in Cattle of district Karak and Kohat KPK, Pakistan

Season	Karak (+/total)		Kohat (+/total)		Total (%)
Winter	3	400	6	400	1.125
Spring	12	200	24	200	9
Summer	79	400	84	400	20.375
Autumn	17	200	12	200	7.25
Total	111	1200	126	1200	9.875

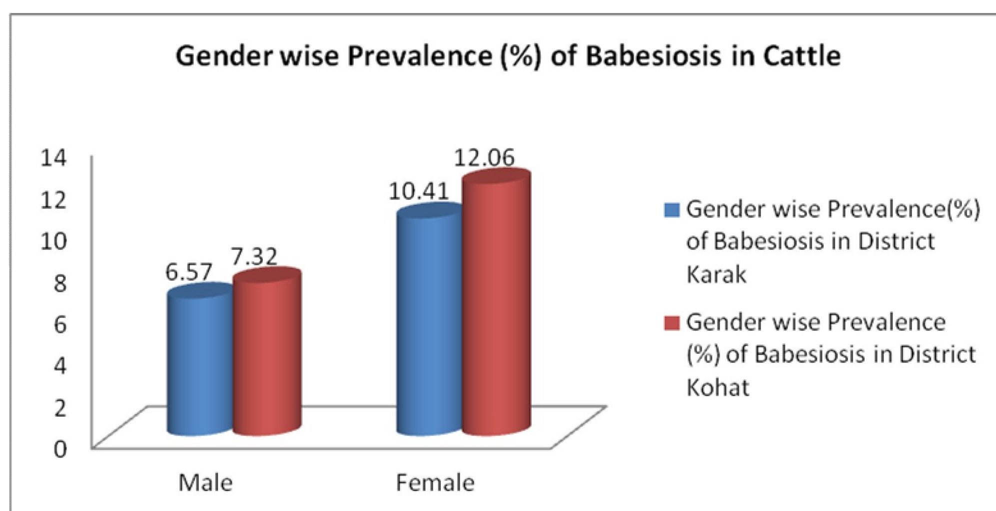


Fig 2. showing the gender wise distribution of Babesia spp in cattle.

3.1. Detection of Babesiosis by Polymerase Chain Reaction (PCR)

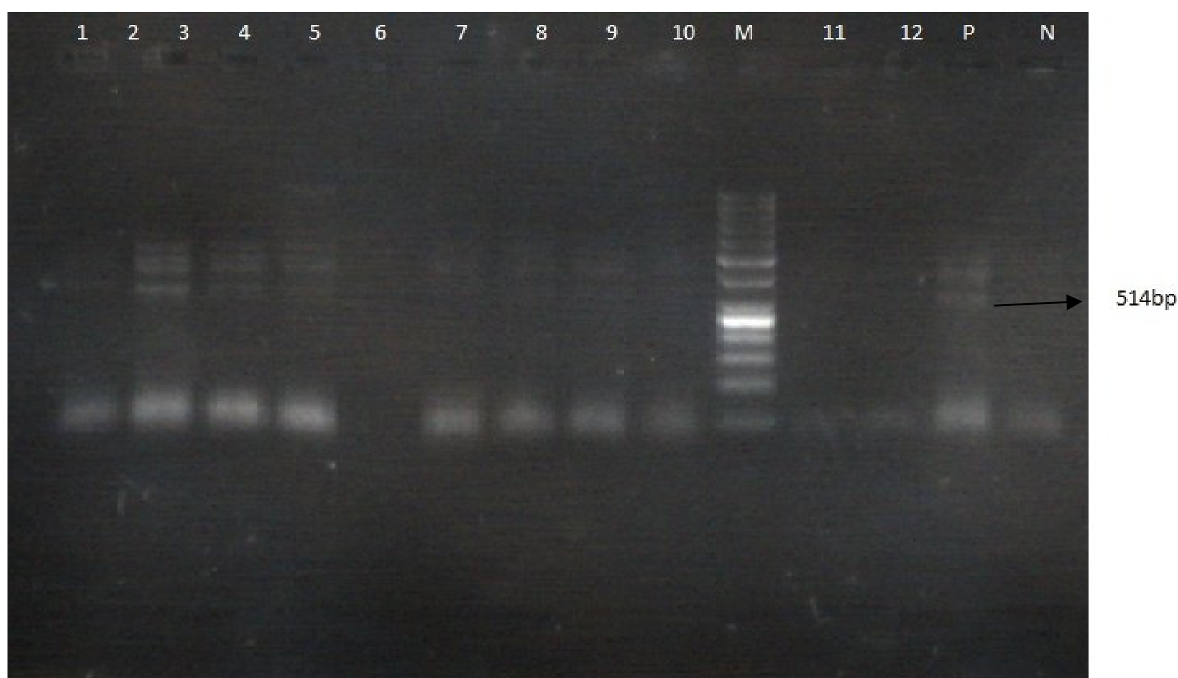
The six hundred samples were randomly examined from the cattle population. The prevalence rate of Babesiosis was 27.5% (165/600) by PCR; among these 24% (72/300) prevalence was noted in district Karak and similarly 31% (93/300) in district Kohat.(table.3 and Fig 3) The prevalence of *Babesia* species in cattle i.e calves and cows was found 20.6% (62/300) and 24.3% (103/300) through PCR technique respectively while 7.7% (23/300) and 12% (36/300) by microscopy technique was detected (table.4). A total prevalence of Babesiosis in Cattle in two districts of Khyber Pakhtunkhwa, Pakistan was 27.5% (165/600), among these *B.bovis* 9.6% (29/300), *B.bigemina* 6% (18/300) and mixed infection 15.3% (46/300) were noted in areas of district Kohat while *B.bovis* 7.3% (22/300), *B.bigemina* 4.6% (14/300) and mixed infection 12% (36/300) in district Karak. The incidence of *Babesia* species in the cattle population of district Kohat was noted higher than that of district Karak.

Table 3. Area wise prevalence of *B.bovis* and *B.bigemina* in cattle by PCR in two districts of KPK, Pakistan

Location	Indicator	*B.bovis* (%)	*B.bigemina* (%)	Mixed infection (%)	Total (%)
Kohat	**Calves (n=150)**	12 (8)	7 (4.6)	18 (12)	37 (24.6)
	Cows (n=150)	17 (11.3)	11 (7.3)	28 (18.6)	56 (37.3)
Subtotal (300)		29 (9.66)	18 (6)	46 (15.3)	93 (31)
Karak	**Calves (n=150)**	7 (4.6)	5 (3.3)	13 (8.6)	25 (16.6)
	Cows (n=150)	15 (10)	9 (6)	23 (15.3)	47 (31.3)
Subtotal (300)		22 (7.3)	14 (4.6)	36 (12)	72(24)
Grand Total (%)		**51(8.5)**	**32 (5.3)**	**82 (13.6)**	**165(27.5)**

Table 4.Prevalence of *B.bovis* and *B.bigemina* in Cattle by microscopy and PCR

Indicator	Calves(n=300)		Cows(n=300)	
	Blood smear (%)	**PCR (%)**	**Blood smear (%)**	**PCR (%)**
B.bovis	7(2.3)	19(6.3)	11(3.66)	32(10.6)
B.bigemina	4(1.3)	12(4)	7(2.3)	20(6.6)
B.bovis/B.bigemina	12(4)	31(10.3)	18(6)	51(17)
Grand total	23(7.7)	62(20.6)	36(12)	103(34.3)



Fig;3. M. showing the 100

M;showing the 100bp DNA ladder

P. for positive control of B.bovis

C.negative control of B.bovis

Lane 1,2,3 and 9 showing positive band of 514bp of B,bovis

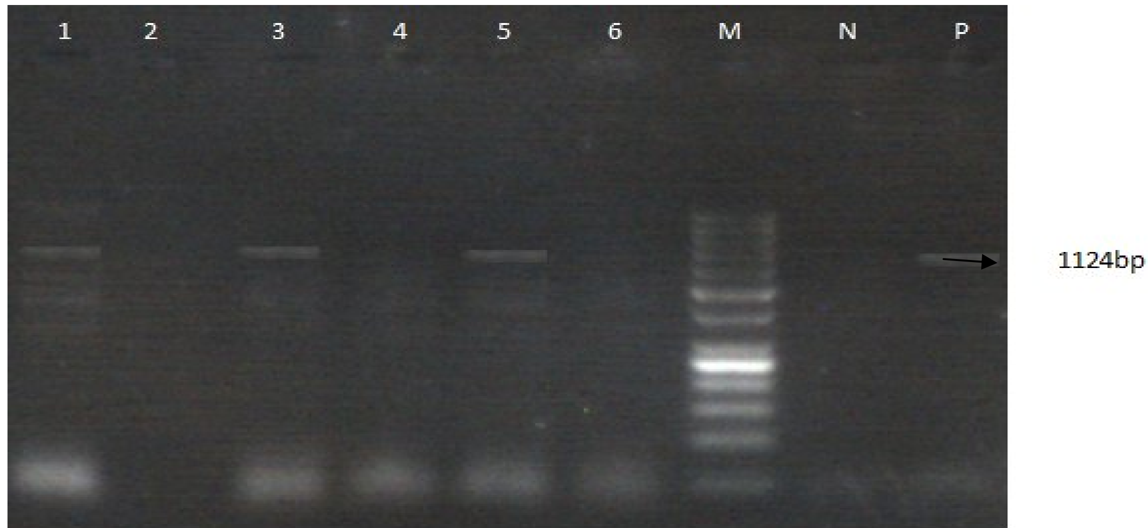


Fig 4.

M;showing the 100bp DNA ladder

P for positive control of B.bigemina

C.negative control of B.bigemina

Lane 1,3 and 5 showing positive band of 1124 4bp of B.bigemina

Babesiosis is a tick borne disease of domesticated animals throughout the world particularly in the tropical, subtropical and Asian countries causing high morbidity and mortality as well as economic losses. The microscopy is still consider the most cheapest and reliable diagnostic technique in cattle / livestock that have adopted in the rural community from a very long time. In the present study it was revealed that Babesiosis was diagnosed in 9.87%(237/2400) cattle in two district of Khyber Pakhtunkhwa, amongst these karak 9.25 % (111/1200) and kohat 10.5 % (126/1200) were recorded by microscopy which seem to be that the cattle having heavy babsiosis infection. This technique is not very successful in detecting the Babesia infection in the carrier cattle and also not so sensitive as different species of Babesia may resemble each other morphologically.Which play important role in the transmission of the tick borne infection in the [17]. Sergent et al. (1945) in one of the study in Algeria was reproduced that the prevalence of babesia in cattle was 29% .In contrast to our study may be due to managemental and hygienic situation in areas however the low level of incidence of Babesia species detected in the carrier cattle may be due to fluctuations of parasitemia that occur during the chronic phase of infection by Babesia species in cattle [18,19,20] and the low numbers of intra-erythrocytic infection in bloodstream of Babesia carriers[21]

During the study, the high prevalence of Babesiosis was recorded in the summer 20.375% (163/800) followed by spring 9% (36/400), autumn 7.25% (29/400) and the lowest was seen in the winter 1.125% (9/800). Similar finding was reported by Bishop et al. (1993) that geographic, season and vector specificity were help in identification of the babesia species in cattle [22]. In the current study, the PCR method was applied in detection of babesia infection both in clinical as well as in carrier cattle randomly and found that it was possible to diagnose the infection in the blood in cattle population. The six hundred samples were randomly examined from the cattle population. The prevalence rate of Babesiosis was 27.5% (165/600) by PCR; among these 24% (72/300) prevalence was noted in district Karak and similarly 31% (93/300) in district Kohat. The DNA was extracted from all the samples in very clean and hygienic environment and all the samples DNA were amplified. The sensitivity of the PCR was 100% and specificity was 96% as compare to microscopy 43.3% sensitivity in detection of Babesia bovis in cattle in the present study The lowest detection limit of the PCR was 2-3 parasites per ml of infected blood, which corresponds with a parasitemia of 0.000048% (Christine et al. 1995) while our results

demonstrate that PCR method detects infection/ Babesia in very low level of 1-2 parasites in blood in carrier cattle. In the current study the performance for detection of babesia in cattle in the blood of microscopy techniques was lower than that of PCR assay. Similar report was presented by Irvin 1987 that it was very difficult to make distinguishing in the parasites i.e. Theileria and babesia species on the basis of the morphology, size, and developmental phases. The difficulty was arise when the mixed kind of infection occur under field conditions, it is important to be able to distinguish the infections caused by different species of B.bovis throughout the world.

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