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Molecular Detection of *Babesia bovis* and *Babesia bigemina* in Vector Ticks, *Boophilus microplus* Collected from Domesticated Cattle in Kohat and Karak Region by using PCR Assay

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Babesiosis is a tick-transmitted disease of veterinary and medical importance causes significant morbidity and mortality in cattle population of Kohat region of Khyber Pakhtunkhwa. The study was designed to analyze/detect the *Babesia* species in vector ticks by PCR method and to identify the prevalent tick species as per standard procedure of taxonomic keysin kohat region of Khyber Pakhtunkhwa Pakistan. A total1032 tick Samples of ticks were examined from different body parts of 800 cattle (cows and calves) of district Karak and Kohat Khyber Pakhtunkhwa Pakistan and were identified as per taxonomic keys and 311 female vector ticks (*Boophilus microplus*) collected from 78 cattle (39 Calves and 39 Cows) were screened through PCR for the detection of *Babesia* species using species specific primers andFemale vector ticks, *Boophilus microplus* were found infected with *Babesia*species. The *Boophilus microplus* was found 36.2% (374/1032) followed by *Hyalommaanatolicum* 33.33% (344/1032), *Rhipicephalussanguineus* 20.6% (213/1032) and the lowest was *Boophilus annulatus* 9.78% (101/1032). Prevalence of *B.bovis* was found 11.2% (35/311), *B.bigemina* 6.7% (21/311) and mixed infection 15.1% (47/311) respectively in vector ticks. The study reveals that Babesiosis is endemic in the study areas and needed proper preventive measure to minimize its prevalence.

Key words: PCR, *B.bovis, B.bigemina* and *Boophilus microplus.*

Babesiosis is a tick bovine transmitted disease of cattle caused by two intraerythrocytic protozoan parasite of the genus *Babesia* (Family: Babesiidae) namely *Babesia bovis* (*B. bovis*) and *Babesia bigemina* (*B. bigemina*)[1].

Ticks (*Boophilus* species) are the major vector for the transmission of Babesiosis[2], which are widely spread in tropical and subtropical countries [3] particularly in Pakistan, Bangladesh and India as the environmental conditions favor the growth and development of many tick species[4].

The cattle tick, *Boophilus microplus* (hard ticks) belonging to the family Ixodidae is predominant in Asia and was found to infest 28.3% cattle[5]. Tick infestation increased in summer as ticks activities become higher in damp and hot environment[6]. Any factor affecting the survival of the tick vectors will affect the risk of Babesiosis occurring[7].

The epidemiological details of Babesiosis are important on the dynamics of transmission by the vector ticks for the embellishment of adequate control strategies[8]. Serological techniques such as the complement fixation test (CFT), the indirect

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hemaglutination (IHA) test, the latex agglutination test (LAT), the indirect fluorescent antibody test (IFAT), and the enzyme-linked immunosorbent assay (ELISA) may also be used but due to interspecies cross reactions, it is difficult to differentiate between post exposure and present infection[9]. Polymerase chain reaction (PCR), which is more sensitive and specific technique, offers an alternative approach for the diagnosis of Babesiosis[10]. PCR-based techniques were used in order to estimate the rate of *B. bovis* and *B. bigemina* infection in *B. microplus females* (vector ticks) collected from infested cattle of various age groups.

MATERIALS AND METHODS

Ticks collection and Identification

A total of 800 cattle (Calves and Cows) were examined for tick infestation from April-July 2012 (200 cattle per month) in district Karak and district Kohat and 1032 ticks were collected. Tick samples were collected with the help of forceps without damaging their body parts and preserved in 70% alcohol. The collected specimens were rinsed in distilled water to make them free from the preservative and boiled in 10% potassium hydroxide (KOH) solution for 30 minutes to remove excess of chitin. Cleared specimens were treated with 10% glacial acetic acid for 5 minutes to remove the traces of KOH, washed with distilled water and stained with Geimsa for 2 minutes. Then these were again washed to remove excessive stain. Stained specimens were dehydrated through different percentages of ethyl alcohol i.e. 30, 50, 70, 80, 90% and absolute alcohol. Specimens were cleared in immersion oil. Ticks were examined under the microscope and identified as per literature method.

DNA Extraction

DNA extraction was performed using GF-1 Nucleic Acid isolation Kit (Vivantus USA) according to the manufacturer protocol.

DNA Amplification

DNA of *Babesia* was Amplified using the primer sequences described by Guido *et al.*, 2002.The reaction mixture for a single reaction was consisted of 10X PCR Buffer 2.0¼l; $MgCl_2$ (25mM) 2.5¼l; dNTPs (10mM) 1.0¼l; (P_1) forward Primer (10pmol) 1.5¼l; (P_2) reverse Primer (10pmol) 1.5¼l; dH_2O 8.5¼l; *Taq.* DNA Polymérase (2U/ml) 1.0¼l; Extracted DNA 2.0¼l.Briefly, PCR program was started with initial denaturation at 94ºC for 5 min, and 35 cycles of 94ºC for 30 sec, 50ºC for 30 sec, 72ºC for 45 sec and final extension at 72ºC for 10 minutes.

Gel electrophoresis

0.5X TBE buffer was used in preparation of 2g agarose gel with ethidium bromide (1mg/ml) as per standard protocol.10ul PCR product with 5ulloading dye was electrophorsed and seen through transilluminatorand observed 541 bp products of *B. bovis* and 1,124 bp products of *B.bigemina.*

RESULTS AND DISCUSSION

By tick prevalence

The *Boophilus microplus* was found 36.2% (374/1032) followed by *Hyalomma anatolicum* 33.33% (344/1032), *Rhipicephalus sanguineus* 20.6% (213/1032) and the lowest was *Boophilus annulatus* 9.78% (101/1032) similarly it was found that all the above mentioned ticks species were slightly higher in district Kohat than Karak (Table 1, Fig.1 A and B).

Identification of *Babesia* species from Vector Ticks by PCR

311 female *Boophilus microplus*ticks were collected from 78 cattle and were randomly screened through PCR. DNA were extracted and amplified with different reference primers. Female ticks, *Boophilus microplus* were found infected

Table 1. Prevalence of vector tick species

Areas	No. of ticks	*Boophilus* microplus (%)	*Rhipicephalus* Sanguineus (%)	*Boophilus* annulatus (%)	*Hyalomma* anatolicum (%)
Karak	468	175(37.39)	98(20.94)	42(8.97)	153(32.69)
Kohat	564	199(35.28)	115(20.39)	59(10.28)	191(33.86)
	1032	374 (36.2)	213(20.6)	101(9.78)	344(33.33)

Statistical analysis: General ANOVA, $p < 0.004$

with *Babesia* species. Prevalence of *B. bovis* was found 11.2% (35/311), *B. bigemina* 6.7% (21/311) and mixed infection 15.1% (47/311) respectively in vector ticks (Table .2, Fig. 2, 3 and 4).

Identification of *Babesia species* from vector ticks collected from calves

In the female ticks that collected from 39 calves the prevalence rate of *B. bovis* (10.2%) was found higher than *B. bigemina* (5.7%). Mixed infection rate of both of the species was found 12.1% while the frequency of uninfected female ticks was found 71.7% (Table. 3).

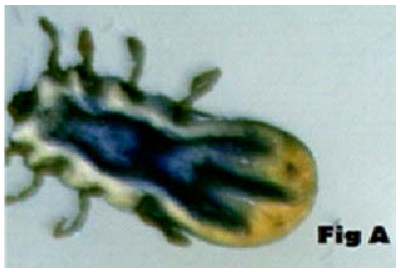


Fig. 1. (A) Male tick (*Boophilus microplus*); (B) Female tick (*Boophilus microplus*)

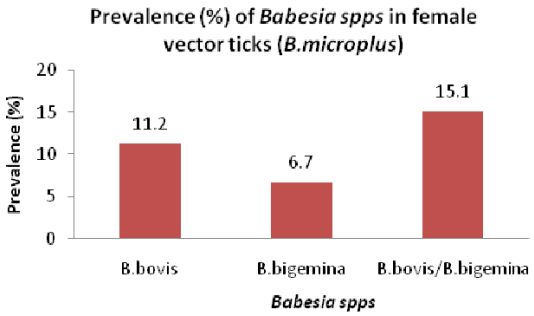


Fig. 2. Number of *Boophilus microplus* Female infected with *Babesia spps*

Identification of *Babesia* species from vector ticks collected from cows

In the female ticks that collected from the cows the prevalence rate of *B. bovis* (12.2%) was higher than *B. bigemina* (7.7%). Mixed infection rate was found 18.06% while the frequency of uninfected female ticks was found 61.9% (Table. 4).

Table 2. Number of *Boophilus microplus* Female infected with *B. bovis* and *B. bigemina* detected by PCR in 311 ticks collected from 78 cattle

Female	*B.bovis*(%)	*B.bigemina*(%)	*B.bovis/B.bigemina*(%)	Negative (%)
Boophilus microplus (n=311)	35(11.2)	21 (6.7)	47 (15.1)	208 (66.8)

Table 3. Number of Boophilus microplus Female infected with B.bovis and B.bigemina detected by PCR in 156 ticks collected from 39 calves

	Calves infection (n=39)			
Female	*B.bovis*(%)	*B.bigemina*(%)	*B.bovis/B.bigemina*(%)	Negative (%)
Boophilus microplus (n=156)	16(10.2)	9 (5.7)	19 (12.1)	112 (71.7)

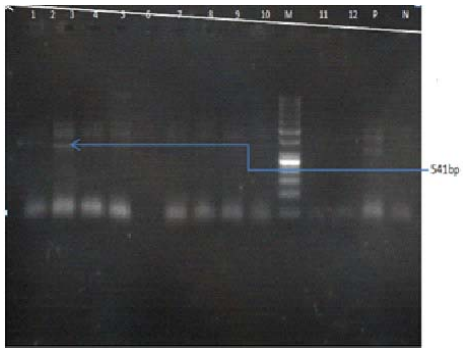
Table 4. Number of Boophilus microplus female infected with B.bovis and B.bigemina detected by PCR in 155 ticks collected from 39 cows

	Cows infection (n=39)			
Female	*B.bovis*(%)	*B.bigemina*(%)	*B.bovis/B.bigemina*(%)	Negative (%)
Boophilus microplus (n=155)	19(12.2)	12(7.7)	28 (18.06)	96 (61.9)

Information regarding the prevalence of *Babesia spps* in potential vector ticks of the region is essential for the epidemiology of Babesiosis.In the present study, the identified species are in compliance with previous reports and the most commonly infested tick species in difeerent localities of district Karak and district Kohat were*Boophilus microplus* (36.2%), *Rhipicephalussanguineus* (20.6%), *Boophilusannulatus* (9.78%), *Hyalommaanatolicum*(33.33%)and same were found by[6, 11] from Peshawar Khyber Pakhtunkhwa Pakistan. Similar findings were observed from other regions of Pakistan[12-14]. Small difference in ticks prevalence and different tick species may be due to area, vegetation, humidity and rain fall differences.

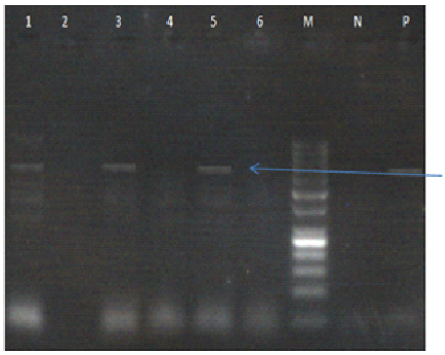
In the present finding, Out of 311 female *Boophilus microplus* was collected from 78 cattle were randomly screened and DNA were extracted and amplified with different reference primers. It was found that female *Boophilus microplus* were found infected with *Babesia species* and isolated through PCR which was recorded that *B. bovis* 11.2% (35/311), *B. bigemina* 6.7% (21/311) and mixed infection 15.1% (47/311) were found respectively. Oliveira-Sequeira *et al*.,[8] reported that PCR diagnostic technique for *B. bovis* and *B. bigemina* in ticks and cattle blood were very effective in approaches However, their *B. bigemina* primer set was directed at a *B. bigemina* genomic DNA fragment of unknown identity and it was reported in their study that the primer sequence had a mismatch with the intended target DNA sequence which could be responsible for false negative results.[15] also used a PCR method to detect *Babesia species* in ticks communities in domesticated cattle. As they reported the problem with the Specificity of 18S primers to identify ticks nourishing *B. bigemina* or *B. bovis*.

Babesia spp. infection was found more frequent in female ticks collected from cows as compare to calves, 38.06% and 28.2% respectively which is in contrast to the study of[8].This may be due to difference in the screening methodsas well as due to difference in cattle species from which the vector ticks are collected. Oliveria[8] used microscopic technique for the detection of *Babesia*



M: 1kbp DNA ladder
Lane: 2, 3 and 5 Positive
Lane: 1, 6, 7, 8, 9 and 10 Negative
P: Positive Control
N: Negative Control

Fig. 3. 541bp DNA of *B.bovis*



M: 1kbp DNA ladder
Lane: 1, 3 and 5 Positive
Lane: 2, 4 and 6 Negative
P: Positive Control
N: Negative Control

Fig. 4. 1124bp DNA of *B.bigemina*

*spp*s.in ticks collected from *Bos Taurus* and *Bosindicus*dairy cows and calves.

This study revealed that *B. microplus* females are more frequently infected with*Bebasia spps*. Different authors who worked on the quantitative aspects of *Babesia* transmission also reported that *B. microplus* females are more frequently infected with *Bebasiaspps*[16-18]. Female ticks that became infected on cattle, in which the frequency of infection with *B. bovis* (11.2%) was higher than the frequency of infection with *B. bigemina* (6.7%).With respect to *Babesia species*, the signiûcantly higher number of female ticks that became infected on calves and cows may represent proof that age may play vital role in the interfere in different ways with the infectivity of the different Babesia species for tick.

CONCLUSION

The study reveals that Babesiosis is endemic in the study areas and needed proper preventive measure to minimize its prevalence.

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