Sensitivity and Specificity of PCR & Microscopy in detection of Babesiosis in domesticated cattle of Khyber Pakhtunkhwa, Pakistan.

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ABSTRACT.

Background: Babesiosis is a tick-transmitted disease of veterinary and medical importance causes significant morbidity and mortality in cattle. This study is proposed to analyze/detect the active infection of Babesiosis in cattle by PCR and microscopic screening and to determine the sensitivity and specificity of the both diagnostic techniques in detection of Babesiosis in Khyber Pakhtunkhwa Pakistan **Methods**: Six hundred blood samples of clinically suspected cattle were collected from veterinary hospitals of district Karak and Kohat Khyber Pakhtunkhwa Pakistan. Thick and thin smear slides were examined under microscope. DNA was extracted from serum and was amplified by PCR using species specific primers. Amplified product was analyzed after electrophoresis under UV transilluminator. **Results:** Overall prevalence of *Babesia* was 27.5% (165/600) in cattle by PCR amongst these 20.6% (62/300) in calves and 34.3% (103/300) in cows. Similarly overall prevalence was 9.83% (59/600) by microscopy, amongst these 7.7% (23/300) in calves and 12% (36/300) in cows. Of these *B. bovis* was detected in 6.3% in calves ,and 10.6% in cows and *B.bigemina* 4% in calves and 6.6% in cows by PCR while the mixed infection was 10.3% in calves and 17% in cows. The sensitivity of PCR was 100% and specificity was recorded 93.67% in detection of B.bovis as compare to Microscopy sensitivity was 36.84% and specificity 100% was observed. **Conclusions:** The study reveals that PCR is more sensitive technique in diagnosis against Babesiosis as compare to microscopy and recommended it for field application in Khyber Pakhtunkhwa Pakistan. So that proper preventive measure against this disease should be taken to increase the productivity of domesticated cattle.

Key Words: *Babesia,* cattle, PCR, Ticks, Khyber Pakhtunkhwa

1.INTRODUCTION

Babesiosis is economically the most important tick-borne disease of cattle (Bock et al., 2004), which causes extensive monetary loss to the cattle industry in the developing countries (Thammasirirak et al., 2003). The economic impact of babesiosis also occurs in other domestic animals, including horse, sheep, goats, pigs and dogs (Chaudry et al., 2010). Clinical signs of this disease are characterized by fever, anemia and hemoglobinuria in the infected cattle (Iseki et al., 2010) which may causes abortion in pregnant ones and reduced fertility in males especially bulls (Zulfiqar et al., 2012).

Babesiosis is caused by two intra-erythrocytic protozoan parasite of the genus *Babesia* (Family: *Babesiidae*) namely *Babesia bovis* (*B. bovis*) and *Babesia bigemina (B. bigemina)* (Iseki et al., 2010). Generally *B. bovis* is more pathogenic than *B. bigemina* (Chaudhry et al., 2010), however both can cause clinical disease and carrier infections (Goff et al., 2008). Ticks (*Boophilus* species) are the major vector for the transmission of both these spp. of *Babesia* (Chaudry et al., 2010), which are widely spread in tropical and subtropical countries particularly in Pakistan, Bangladesh and India as the environmental conditions favor the growth and development of many tick species (Irshad et al., 2010) *Babesia* in infected animals can be detected by microscopic examination of blood smears stained with Giemsa (Altay et al., 2008), but this method shows low sensitivity and time consuming (Adham et al., 2009). Serological methods (ELISA) may also be used but due to interspecies cross reactions, it is difficult to

differentiate between post exposure and present infection (Adham et al., 2009). Polymerase chain reaction (PCR), which is more sensitive and specific technique, offers an alternative approach for the diagnosis of babesiosis (Zulfiqar et al., 2012).

This study was aimed to determine the sensitivity and specificity of PCR and Microscopy diagnostic techniques in diagnosis of Babesiosis in the domesticated cattle in district kohat and karak of Khyber Pakhtunkhwa province Pakistan.

2. MATERIALS AND METHODS

2.1 sample collection.

Six hundred Blood samples were collected randomly from the clinically suspected cattle for babesiosis and were labeled the date of collection, sex, age and the areas where the sample were collected. The samples were transported in very sterilized container and stored at $-20^{o}C$ in Molecular Parasitology and Virology Laboratory Department of Zoology, Kohat University of Science and Technology Kohat Khyber Pakhtunkhwa Pakistan. Clinical data was recorded on designed proforma.

2.2 Microscopy

Thick and thin blood smears were fixed with absolute methanol for one minute and was stained by Giemsa stain. Stained slides were observed under 100x magnification using microscope (Olympus Japan) to detect *B. bigemina* and *B. bovis*. Pictures of positive slides were taken. *B.bigemina* is larger in size and having paired structure at an acute angel to each other. *B.bovis* is smaller in size and having paired form at an obtuse angel to each other. However it is hard to differentiate the two species by microscopic examination.

2.3 MOLECULAR DETECTION
2.4 DNA isolation

DNA was isolated from the whole blood using GF-1 Nucleic Acid isolation Kit (Vivantus USA) according to the manufacturer procedure.

2.5 DNA amplification and Detection

DNA was amplified for *B. bigemina* and *B. bovis* accordingly mentioned by Guido et al. (2002). 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. Finally the amplified DNA was analyzed under UV transilluminator after electrophoresis on 1% agarose gel, and compared with 100bp DNA ladder (Fermentas USA), used as size marker.

2.6 Sensitivity and specificity of PCR and microscopy

Sensitivity values for PCR were obtained using the following correlations.

Sensitivity= true positive/ true positive + false negative X 100
Specificity as calculated as follows
Specificity = true negative/ true negative + false positive X 100 (Anderson et al., 1989).

3. RESULTS

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.1)

The prevalence of Babesia species in cattlei.e calves and cows was recorded 20.6% (62/300) and 24.3% (103/300) through PCR technique respectively. Similarly 7.7% (23/300) and 12% (36/300) by microscopy technique was detected. In the present study the aetiological agent i.e. species of the Babesia were detected I cows and cows and it was found thatB.bovis 2.3% (7/300) followed B.bigemina1.3% (4/300) and mixed infection 4% (12/300) through microscopy while B.bovis 6.3% (19/300), B.bigemina 4% (12/300) and mixed infection 10.3% (31/300) (table.2)

Similarly 300 random sampling were carried by both of the techniques in cows which shows *B.bovis* 3.68% (11/300), *B.bigemina* 2.3% (7/300) and mixed infection 6% (18/300) through microscopy while 10.6%(10/300), 6.6% (20/300) and 17% (51/300) were reported by PCR respectively(table3)

4.Discussion

Babesiosis is one of the most prevalent diseases, affecting cattle and causing huge losses to the cattle industry (Ziapour et al., 2011). The prevalence of babesiosis in cattle is about 1.2 billion in many countries of the world including Asia, Australia, Africa, South and Central America and the United States (Zulfiqar et al., 2012) while in Pakistan about 5.5- 42.8% cattle and buffaloes are exposed to this disease (Niazi et al., 2008). In order to decrease the monetary losses by the parasite it is mandatory to diagnose and treat Babesiosis well in time. The most commonly used methods are microscopic examination of blood smears stained with Giemsa and PCR which is more specific and sensitive technique for the detection of *Babesia* spp. present in blood of animals (Shahnawaz et al., 2011; Mesplet et al., 2011).

We found high prevalence, 27.5% of *Babesia spp.* (8.5% were positive for *B. bovis* , 5.33% was *B. bigemina* and mixed infection was 13.67% through PCR while 9.83% were positive by microscopic examination. Similarly results were found by other

researcher from different areas of Pakistan like Qadirabad (Chaudhry et al., 2010), Kasure (Durrani and Kamal 2008), North Iran (Ziapour et al., 2011), Brazil (Oliveira-Sequeira et al., 2005), Egypt (Adham et al., 2009), Tunisia (Ghirbi et al., 2008), Kalubyia Governorate (Talkhan et al., 2010) and Southern Punjab (Zulfiqar et al., 2012). Moreover, small differences in the prevalence rate may be due to environmental factors of the areas, sample size and cattle breads under study.

Our results also showed that cows were more infected by *Babesia* spps as compared to calves which are in contrast with the findings of Niazi et al., (2008), Zulfiqar et al., (2012) Atif et al., (2012) who found more infections in calves in Punjab. This may be due to poor managemental and environmental condition. More infestation of calves may be due to softer and thinner skin of young animals which help the vector (ticks) to transmit the disease easily.

In the present study it was found that PCR was more sensitive than microscopy similar finding was reported in detection method of protozoa which depends on several factors including the species whether they are capable of human infection (Li et al., 2004) and adopted the microscopy procedure for the identification of protozoa by Ho et al., 1995; smith et al., 2002) while PCR identification was more sensitive than microscopic examination as reported by Jiang et al., 2005.

Table No.1 .**Detection of Babesiosis in district Karak and Kohat Khyber Pakhtunkhwa, Pakistan by PCR**

Districts	+ive Samples/total	Prevalence (%)
Kohat	93/300	31%
Karak	72/300	24%
Grand total	165/600	27.5%

Table 2: **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%)	12(4%)	103(34.3%)

Table No.3 sensitivity/ specificity of PCR and microscopic techniques against the detection of Babesiosis in domesticated cattle in Khyber Pakhtunkhwa Pakistan.

Parasites (n)	PCR			Microscopy		
	True+ive	Sensitivity%	Specificity%	True +ive	Sensitivity%	Specificity%
B.bovis	19	100	93.67	7	36.84	100
B.bigemina	12	100	96	4	25	100
B.bovis/B.bigemina	31	100	89.66	12	11.42	100

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