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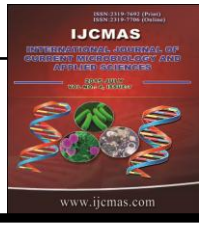


**Original Research Article**

# Prevalence and Molecular Detection of Babesiosis in the Slaughter Animals of Peshawar Khyber Pakhunkhawa Pakistan

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Prevalence, Babesiosis, ticks infestation, polymerase chain reaction (PCR)

**A B S T R A C T**

Babesiosis is most prevalent in the sub tropical and tropical regions of the world, causing huge losses to valuable breeding animals stock due to slaughtering. A total of 100 blood samples from tick infested cattle's were analyzed for *Babesia bovis* and *Babesia bigemina* by Polymerase chain reaction and microscopy. Out of 100 samples 73 were cows and bulls and 27 were Buffaloes. In Buffaloes no positive male case were found for both species in Polymerase chain reaction. In microscopy only 24 positive cases were diagnosed in which 17 cases comprising of 4 cows and 13 bulls but in Polymerase chain reaction 37 positive cases were diagnosed in which 5 were of mixed type and 11 samples were found positive for *B. bovis* and 21 samples for *B. bigemina*. Bulls were more infected as compared to female. In this study we concluded that *B. bigemina* is more prevalent in slaughter animals than *B. bovis*. Overall results showed that PCR is more sensitive tool for the Babesiosis diagnosis.

## Introduction

Babesiosis is most prevalent in the sub tropical and tropical regions of the world. It is more prevalent after trypanosomiasis worldwide. Babesiosis causing huge mortality and morbidity, especially in the world under developing countries including Pakistan. There are more than 100 Babesia species infecting a wide range of mammals (Hunfeld et al., 2008.Collet, 2000 and Ruheta et al., 2007). The two major Babesia species that causes huge mortality and morbidity in cattle population are *Babesia bovis* and *Babesia bigemina.* Babesiosis is fatal for cattle population when no health facilities were provided (Radostitis et al., 2000 and Durrani and Kamal, 2008). All over the world 1.2 billion while in Pakistan 5.5-42.8% cattle are at high risk of babesiosis infection (Kim, 2007and Niazi, 2008). Babesiosis has reducing effects on milk, beef and meat production. It has adverse effect on the cattle trade internationally (Bock, 2004 and Mosqueda, 2012). Babesiosis is characterized by

anemia, fever and haemolysis. It also causes infertility in males and miscarriage in females. *Babesia bovis* is more pathogenic as compared to *Babesia bigemina* (Iseki, 2010). Babesiosis in infected animals can be diagnosed by microscopy technique but this method is time consuming and having low sensitivity. Polymerase chain reaction presents a substitute approach for the diagnosis having high sensitivity and specificity (Zulfiqar et al., 2012 and Centers for Disease Control and Prevention, 2013). Keeping in view the loss in international cattle trade and loss of valuable breeding stock of animals due to slaughtering, the present study was intended to be acquainted with prevalence and molecular detection of babesiosis in the slaughter of Peshawar Khyber Pakhunkhawa Pakistan.

## Material and Methods

### Study Area

Blood samples were collected from the different slaughter animal houses of Peshawar, which is located in large valley near eastern end of Khyber Pass close to pak Afghanistan border. Geographically it is 34° 0′ 28″ North, 71°34 ′ 24″ East, having temperature range 25-40c̊. The winter rain fall level is higher than summer.

### Specimen collection

A total of 100 blood samples were collected from May, 2011 to Nov, 2011 in sterilized vacationer having capacity of 5ml from jugular vein, including 27 buffaloes, 73 bull and cows after the approval of ethical Committee University of Peshawar. Data with characteristics of the animals that were specie, gender and tick infestation along with symptoms were reported.

### Sample Processing

The blood samples were processed through microscopy, Polymerase chain reaction and finally through Gel electrophoresis.

### Microscopy

Thin blood smears were prepared on a clean glass slide, Fixed with Methanol for 1 minute and Stained with Giemsa stain (1:10 dilution) for 30minutes. After staining, slides were washed with tape water and dried in air. Finally the stained slides under 100x magnification of Olympus microscope were observed for *Babesia bigemina* and *Babesia bovis*, by studying their morphological individuality described by (Soulsby., 1982).

### DNA Extraction and Amplification (PCR)

DNA was extracted through the rapid boiling method as described by Foley et al (Foley, 1992). The extracted DNA was stored at -20c°. For the DNA amplification of *Babesia bigemina* and *Babesia bovis* Polymerase chain reaction was used. Two separate reaction mixture were prepared having extracted DNA 2.0 ul, dNTPs(10mM) 1.0ul, 10xPCR buffer 2.0ul, $Mgcl_2$ (25mM) 2.5ul, Taq DNA polymerase (2u/ul) 1.0ul, forward and reverse primer each 1.5ul (Figueroa, 1992), having sequence as provided by different researchers (Allsopp, 1993 and Guido, 2002) as in Table 1. The Thermal cycler was set for 30 cycles. Denaturing of samples was done at 94C° for 5 minutes, annealing at 94C° for 30 second, 60C° for 45 seconds and extension at 60C° for 7 minutes (Figueroa, 1992).

### Gel Electrophoresis

Gel electrophoresis of the PCR product was carried out by using 0.7% agarose gel in 10x TBE (10 times) working solution with Ethidium bromide (1ug/ml) stain. A DNA ladder 50base pair mixed with DNA loading

buffer was also used. 541 bp and 1124 bp DNA products of *Babesia bovis* and *Babesia bigemina* respectively were visualized by UV transilluminater.

**Data Analysis**

Statistical analysis was performed by using ANNOVA at significance level $P<0.05$ statistically and one-sample T test using by using Statistic version 9.

**Prevalence Rate**

The prevalence rate was determined by the following formula, Prevalence Rate = (No. of parasite detected in blood samples/Total no. of blood samples examined) ×100

**Results and Discussion**

The overall prevalence of babesiosis was 61% (61/100) in the slaughter animals of Peshawar recorded by microscopy and PCR. Among these 61%, 24% and 37% were positive by microscopy and PCR respectively during this study. In microscopic technique Out of 24 positive Babesia samples 16 were of Babesia bigemina having 3 cows and 13 bulls and 8 of Babesia bovis including 8 buffaloes (6 female and 2 male), indicating that Babesia bigemina is more prevalent in slaughter animals of Peshawar than Babesia bovis as presented in Table 2.

The PCR results showed that out of 100 blood samples 37 samples were positive having 5 mixed type infection. In 37 samples 7 bulls and 2 cows were infected by *Babesia bovis* while 14 bulls and 1 cow was infected with *Babesia bigemina* while only 2 female Buffaloes were infected with *Babesia bovis* and 6 female Buffaloes were infected with *Babesia bigemina.* The highest prevalence of *Babesiosis* was found in the Bulls then cows; also incidence rate of Babesiosis was greater in cattle's then in Buffaloes. Mixed infection was also found in 5 animals by PCR having 3 males and 2 females as shown in table 3. No positive male buffalo case was observed for both species in PCR assay.

In the present study stated that in out of 100 samples 24% were detected by microscopy and 32% were detected by PCR while the mixed infection was observed 5% as shown in Figure No. 1. It was also observed that all slaughter animals from which Blood were collected have ticks infestation.

For the prevalence of Babesiosis ticks of that region are instrumental from the epidemiological point of view. Diseases transmitted by the ticks are economically important throughout the world (Uilenberg, 1992). Recovered animals are carriers which are important in the transmission by the ticks (Oliveira, 1995). In many countries including South and Central America, Africa, United States and Australia 1.2 billion Babesiosis cases are estimated (Terkawi, 2011).

In Northern areas of Pakistan ticks species such as *Boophilus microplus, Hypoderma lineatum, Hyalomma a.a.natolicum*, and *Hyalomma aegyptium* in cattle's were reported (Ali, 1986). Goff and their fellows showed high prevalence of Babesiosis both in calves and in adult animals (Goff, 2002). Zulfiqar and other scientists., (2011), worked on Babesia bovis in large ruminants of southern Punjab and their effect on different aspects of serum biochemical and Hematological profile. They concluded that age of animals, presence of ticks on animals; ticks of dogs in relation to herds are instrumental in *Bovine babesiosis* in large ruminants. *Babesiosis* is acquired during pasteurization and associated to vector tick (Zulfiqar et al., 2012).

**Table.1** Primers used in PCR

| Primer | sequence | Target position on genome | Predicated amplcon size |
|---|---|---|---|
| A | ***Babesia bovis* specific** | | |
| BF1 | CTGTCGTACCGTTGGTTGAC | 675-694 | 541bp |
| BR2 | CGCACGGACGGAGACCGA | 1215-1198 | |
| B | ***Babesia bigemina* specific** | | |
| BF3 | TGGCGGCGTTTATTAGTTCG | 409-428 | 1124bp |
| BR4 | CCACGCTTGAAGCACAGGA | 1532-1515 | |

**Table.2** Species and sex wise data base on Microscopy

| S/No | Species | Positive | Male (%) | Female (%) | Frequency (%) | P Value |
|---|---|---|---|---|---|---|
| 1 | **B.bigemina** | 16 | 13 (81.25) | 3 (18.75) | 66.67 | 0.0662 |
| 2 | **B.bovis** | 8 | 2 (25) | 6 (75) | 33.33 | |

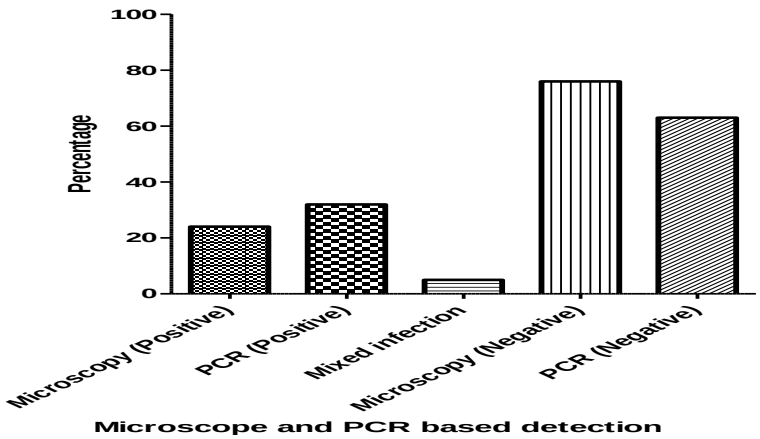
(%) used for percentage, (p) for significance

**Table.3** Species and sex wise data based on PCR

| S.no | Species | male | female | Frequency | Frequency (%) | P Value |
|---|---|---|---|---|---|---|
| 1 | **Babesia bigemina** | 14 | 7 | 21 | 56.76 | 0.0702 |
| 2 | **Babesia bovis** | 7 | 4 | 11 | 29.73 | |
| 3 | **Mixed infection** | 3 | 2 | 5 | 13.51 | |

(%) used for percentage, (p) for significance

**Figure.1** Comparisons of Babesiosis based on Microscopy and PCR



For the diagnosis of Babesiosis most commonly used method is blood smearing in acute cases but in carrier stages where the infection is too low, where more sensitive tools are needed; because Microscopy does not detect the infection in early stage or carrier stage and PCR is most reliable and sensitive method, which diagnose the low grade infections as shown in table 4. In our study some animals were symptom less but their infectivity was shown by PCR indicating *Babesia* species carrier stage which showed the same result as described by Durani and Kamal.,(2008), that the PCR sensitivity in carrier cattle's is 0.000040%. In this research article incidence of *Babesia bigemina* is at high level followed by *Babesia bovis,* as identified by PCR showing 11 cases were of *B.bovis* and 21 of *B.bigemina* as shown in table 3.

Microscopic technique has many drawbacks due to low sensitivity and specificity. Microscopic technique fails to diagnose low level parasitemic cases because of environmental contamination, low quality stain and methanol or having misappropriated concentration, unclean slides or greasy slides and low grade oil emersion (Cadder et al., 1996 and Mesplet et al., 2011). Regarding our results we also observed that PCR efficiency depend on exact amount of using chemicals, even quantity in points may alter the results.

PCR can give false negative results due to absence of target sequence or primer inaccessibility. Absence of target sequence is due to mutation/ deletion of homologous sequence to the primers, denaturation of the DNA during DNA extraction and storage. PCR can give also negative results when amplification failed due to inhibitory sample workings (Jr Barker et al., 1994). PCR has high sensitivity and specificity as compared to microscopy. In our study we concluded that usefulness of smear examination is less then Polymerase chain reaction. Microscopy needs expert eye, expert in blood smear preparation and high quality Giemsa stain, because stain can altered the results.

The overall results showed high incidence of Babesia bigemina, both by microscopy and PCR technique in the slaughter animals of Peshawar Khyber Pakhunkhawa, Pakistan.

Results of this study showed that *Babesiosis* is prevalent in the Peshawar after *Theileriosis* infection, showing incidence of *Babesia bigemina* is more than the *Babesia bovis*. Also our results support the use of PCR as a sensitive tool for the Babesiosis diagnosis. A massive vaccination programmed should be started to reduce mortality rate. There should be professional diagnostic laboratories for the diagnosis of *Babesiosis*. Local Government should use variety of management programs to reduce the diseased cattle's import**.**

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**Competing interest**

Author has declared that no competing interests exist.

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