Direct blood PCR detection of *Babesia bigemina* and its effect on haematological and biochemical profile in crossbred cattle of eastern Haryana

Anita Ganguly*, R. S. Bisla, Indrajit Ganguly†, Harpreet Singh, Vandna Bhanot‡ and S. S. Chaudhri

Teaching Veterinary Clinical Complex, Lala Lajpat Rai University of Veterinary and Animal Sciences, Regional Centre, Karnal-132 001, India.

Received: 01-04-2016  Accepted: 19-07-2016 DOI: 10.18805/ijar.v0iOF.7007

**ABSTRACT**

The present study aimed to diagnose *Babesia bigemina* in naturally infected crossbred cows and to determine its effect on haematological-biochemical profile of host animals. Blood samples from lactating crossbred cows (n=30) between 3-6 years of age and showing clinical signs of babesiosis were collected, with or without anticoagulant, and analyzed for the protozoa by direct smear, direct blood PCR detection of the apical membrane antigen 1 (AMA-1) gene specific amplicon of *B. bigemina* and estimation of haematological and biochemical parameters. Healthy crossbred cows (n=10), examined free from haemoprotozoan infections were included as control. Blood Direct PCR revealed a 448-bp amplified fragment. Out of 150 random blood samples screened, (27/150) 18% were positive under light microscope, whereas direct blood PCR revealed (39/150) 26% samples positive for *B. bigemina*. The result shows higher specificity and sensitivity of PCR test over blood smear examination. The infected group showed significantly (p<0.001) decreased values of TSP (6.12±0.13) and albumin (2.39±0.09) than that of healthy control animals. However, differences in the red blood cell indices (MCV, MCH and MCHC) were non-significant (p>0.05) between the groups indicating normocytic hypochromic anaemia in affected crossbred cattle. Serum samples of infected cows showed significantly (p<0.01) higher values of ALT (78.83±8.95), AST (146.13±7.62), BUN (27.09±1.02), creatinine (1.93±0.1) and TBIL (1.42±0.06) than that of healthy control. A significant decrease (p<0.01) of TEC (3.04±0.19), revealed (39/150) 26% samples positive for *B. bigemina*. The result shows higher specificity and sensitivity of PCR test over blood smear examination. The infected group showed significantly (p<0.001) decreased levels of TSP (6.12±0.13) and albumin (2.39±0.09) than that of healthy control. The standardized blood direct PCR method of the present investigation may be useful for rapid and reliable diagnosis of *B. bigemina* in conjunction with microscopic examination. Moreover, marked changes in haematological and serum biochemical profile observed in *B. bigemina* infected crossbred cows may be useful in understanding disease pathogenesis and undertaking necessary corrective measures.

**Key words:** AMA-1, Babesiosis, *Boophilus microplus*, Haematology, Serum biochemistry.

**INTRODUCTION**

The tick-borne disease babesiosis causes significant morbidity and mortality in cattle worldwide. Protozoan parasites *Babesia bovis* and *B. bigemina*, causative agents of cattle babesiosis, are transmitted by Ixodid ticks *Boophilus microplus* which is wide spread in many tropics and subtropics (O.I.E., 2005). Babesiosis results in considerable adverse economic impact on cattle industry particularly in developing countries including India (McLeod and Kristjanson, 1999). Infections are characterized by high fever, anorexia and dark brown urine (Yeruham et al., 2003). Although cattle infected with parasites are easily detectable during acute infections, however; detection becomes difficult in carrier cattle/chronic infections due to low number of parasites in peripheral blood. Accurate and early diagnosis of babesiosis, especially low-level infections, is important for better management, effective disease control, overcoming economic loss as well as in epidemiological studies (Fahrimal et al., 1992). Although serological methods are widely employed in determining sub-clinical infections; however they suffer from specificity and sensitivity (Durrani et al., 2006; Col and Uslu, 2007; Mahmoud and Abou-Zeina, 2008; Terkawi et al., 2011; Iseki et al., 2010). On the contrary, PCR based technique has been proven to be very sensitive in detecting *B. bovis* and *B. bigemina* infected carrier cattle (Calder, 1996; Salem et al., 1999; Guido et al., 2002, Mosqueda et al., 2012). The present study was aimed at rapid diagnosis of *Babesia bigemina* in naturally infected crossbred cows and to determine its effect on haematological-biochemical profile of host animals.

**MATERIALS AND METHODS**

In the present study, necessary ethical approval was obtained from the Research Review Committee of Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Haryana. Lactating crossbred cows (3-6 years) brought to outpatient department (OPD) of LUVAS Regional...
Centre at Uchani, Karnal during the period of July, 2014 to June, 2015 and showing clinical signs (fever, anaemia, loss of appetite, cessation of rumination, laboured breathing and hemoglobinuria etc.) similar to babesiosis were included in the present study. Crossbred cows (n=30) showing ≥5% parasitaemia constituted the infected group; whereas, ten healthy crossbred cows, free from Babesia (negative both under microscopic examination and direct blood PCR assay) were constituted the healthy control group. The blood samples from both the groups were collected in vials with or without anticoagulant (EDTA). Immediately after collection, blood smears were prepared, stained with Giemsa and the presence of B. bigemina was examined microscopically (Figure 1).

Oligonucleotide primers were designed using online Primer3 software (http://primer3.ut.ee/) and genomic specificity of the primers was tested online using Primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Blood Direct PCR was performed by taking 2µl of whole blood, targeting the AMA-1 gene of B. bigemina, using specific primer pairs (BBF2 5’-ACACCCCTTGGTTACGTCAAG-3’ and BBR2 5’-GGCGTTATGTCACTGGGTCT-3’). Initially PCR assay was standardized on positive samples (blood with ≥10% parasitaemia) using Phusion blood direct PCR kit (Thermo Fisher scientific, India, Pvt. Ltd.) following manufacturer’s instructions. Briefly, PCR was carried out in a thermal cycler (Veriti™, Applied Biosystem) with a final reaction volume of 20 µl containing 2 µl of whole blood, 1 X Phusion blood PCR buffer, 0.5µM of each primer and 0.4 µl Phusion blood II DNA Polymerase. A negative control without template (whole blood) was always included to rule out any PCR carryover. The PCR conditions were initial denaturation at 98°C for 5 minutes; followed by 35 cycles of 98°C for 2sec, 57°C for 30 sec and 72°C for 30 sec; with a final extension step of 72°C for 1 minute. After completion of PCR, tubes were centrifuged at 1000x g for 2 minutes to collect the clear supernatant. Aliquots of 5µl of PCR products (supernatant) were visualized on 1.5% agarose gel (stained with ethidium bromide) and documented under gel documentation system (Gel Doc XR+, Bio-Rad) to confirm the fragment size. Amplified products (two representative samples) were purified and sequenced. All the 30 animals of infected group with varying degree of parasitaemia were screened through standardized direct blood PCR assay. In order to validate the PCR assay and to check its sensitivity, 150 random blood samples of crossbred cattle were screened microscopically as well as through direct blood PCR method. The coagulated blood samples were centrifuged (5000 rpm, 15 min) and supernatant (serum) was collected for biochemical estimations.

Blood collected in anticoagulant vials (1.5 ml approximately) was used for haematological profile and PCR assay. Haematological parameters viz., haemoglobin (Hb- gm/dL), packed cell volume (PCV %), total erythrocyte count (TECx10⁶/µL), total leukocyte count (TLC x10³/µL) and differential leukocyte count (DLC) were analysed as per method described (Schalm et al., 1975). The red blood cell indices like mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated (Cole, 1986).

Total serum protein (TSP), glucose (Glu), calcium (Ca), phosphorus (P), alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), creatinine (Cr), triglycerides (TG), cholesterol, high density lipoproteins (HDL), total bilirubin (TBIL) and direct bilirubin (DBIL) were measured by semi auto analyzer (3000 Evolution, Biochemical Systems International, Italy) employing commercial kits (Siemens Healthcare Diagnostics Ltd. Baroda, India). LDL was estimated by the Friedewald equation (Friedewald, 1972): LDL = TC - HDL - TG/5.0 (mg/dL).

The differences of means of estimated parameters between B. bigemina infected and healthy control groups were compared using Student’s t-test (Snedecor and Cochran, 1994).

RESULTS AND DISCUSSION

The direct blood PCR assay in the present investigations produced 448-bp fragment as expected, pertaining to apical membrane antigen 1 (AMA-1) gene of B. bigemina (Figure 2). Sequencing result further confirmed AMA1 gene specific amplicon. All the animals from infected groups, confirmed positive by stained blood smears, were found to be positive with direct blood PCR assay. When random blood samples were screened under light microscope and direct blood PCR method, 18% (27/150) of samples were positive under microscopic examination. On the other hand, PCR analysis of the samples revealed 26% (39/150)
positive for \textit{B. bigemina}, thus confirming its utility in rapid diagnosis of \textit{B. bigemina} with high specificity and sensitivity especially in carrier animals/subclinical infections. The haematological values of \textit{B. bigemina} infected and healthy control crossbred cows have been presented in Table 1. The infected group showed significantly (p<0.001) lowered values of TEC (3.04±0.19), Hb (4.78±0.27) and PCV (14.53 ±0.87) than healthy control animals. Similar findings had also been reported in \textit{B. bigemina} infection of cattle (Durrani et al., 2006; Sharma et al., 2013). There were no differences (p>0.05) between infected and healthy crossbred cattle pertaining to red blood cell (RBC) indices viz., MCV, MCH, and MCHC (Table 1). Moreover, they were within normal range (Jain, 1993) indicating normocytic hypochromic anaemia (Table 1). However, the type of anaemia largely depends on the disease severity and accordingly various types of anaemia in babesiosis and theileriosis has been reported (Durrani et al., 2006; Sharma et al., 2013, Mbassa et al., 1994; Omer et al., 2002; Ramin et al., 2011; Ganguly et al., 2015). In the present study, normocytic hypochromic anaemia observed in the \textit{B. bigemina} infected crossbred cattle may be attributed to intravascular haemolysis of red blood cells (Pandy and Misra, 1987). Significant increase of TLC (p<0.05) and slight non-significant (p>0.05) increase of lymphocyte counts was observed in infected crossbred animals compare to healthy control group (Table 1). It seems that breakdown of RBCs by \textit{B. bigemina} stimulates the phagocytic cells such as lymphocytes and monocytes to clean up the body from the toxic remnants of ruptured red blood cells. In this context, \textit{Babesia} infection reported to be stimulating the body defence mechanism for production of antibodies against \textit{Babesia} antigen (Guglielmone et al., 1996). Serum samples of \textit{B. bigemina} infected crossbred cows showed significantly (p<0.01) values of ALT (78.83±8.95), AST (104.7±8.32), BUN (24.42±1.34), creatinine (1.93±0.1) and total bilirubin (1.42±0.06) than that of healthy control (Table 2). These results are in agreement with previous reports (Allen and Kuttler, 1981; Camacho, et al., 2005; Hamoda et al., 2014). The increase in enzymes activity and total bilirubin may attribute to severe anaemia that leads to hypoxic and toxic liver damages. Substantial haemolysis in conjunction with hypoxia may lead to hepatic cell degeneration and glomerular dysfunction leading to increase in AST, ALT, BUN and creatinine. Significant (p<0.001) increase of both BUN and creatinine level observed in the present study is in accordance with the result obtained in earlier studies (Camacho et al., 2005; Hamoda et al., 2014). Comparison of lipid profile of healthy and infected groups revealed no significant difference (p>0.05) except at the level of HDL (p<0.05) (Table 2). In the present study, significant decrease of TSP (p<0.005) and serum albumin level (p<0.001) was monitored in the infected group compare to healthy control (Table 2). The decrease of serum albumin value has been found to be associated with the acute phase of many infectious diseases (Allen and Kuttler, 1981). In addition, albumin level may be decreased due to decreased protein synthesis capacity of the affected liver and its excretion in urine as albuminuria in addition to the malnutrition status occurs during the disease (Henley and Judith, 1985). These observed changes in haematological

\begin{table}[h]
\centering
\caption{Mean values\textsuperscript{a} of haematological parameters of crossbred cattle infected with \textit{Babesia bigemina}}
\begin{tabular}{|l|l|l|l|l|l|}
\hline
\textbf{Parameters} & \textbf{Infected Cattle (n=30)} & \textbf{Healthy Cattle (n=10)} & \multicolumn{2}{l|}{\textbf{Reference Range}} & \textbf{P value} \\
\hline
 & \textbf{Mean± SEM} & \textbf{Range} & \textbf{Mean± SEM} & \textbf{Range} & \textbf{Ω} \\
\hline
Hb (gm/dL) & 4.78±0.27 & 2-6.8 & 11.17±0.38 & 9.5-12.9 & 8.0-15.0 & 0.00001** \\
PCV (%) & 14.53±0.87 & 6-20 & 32.9 ±1.18 & 30-40 & 24.0-46.0 & 0.00001** \\
TEC(×10\textsuperscript{3}/µL) & 3.048±0.19 & 1.02-4.49 & 7.05±0.17 & 6.1-7.89 & 5.0-10.0 & 0.00001** \\
TLC (×10\textsuperscript{3}/µL) & 10.630±0.92 & 3.750-28.40 & 5.870±0.332 & 4-6-7.6 & 4.0-12.0 & 0.0219* \\
Lymphocyte (%) & 56.13±2.23 & 30-76 & 50.9±2.0 & 42-60 & 45.0-75.0 & 0.205 \\
Monocyte (%) & 1.1±0.12 & 0-2 & 1.4±0.22 & 1-3 & 2-7.0 & 0.253 \\
Neutrophil (%) & 40.73±2.24 & 20-67 & 46.2±1.94 & 37-56 & 15.0-45.0 & 0.187 \\
Eosinophil (%) & 2±0.18 & 1-5 & 1.5±0.22 & 1-3 & 0-20.0 & 0.158 \\
MCV & 10.630±0.92 & 3.750-28.40 & 5.870±0.332 & 4-6-7.6 & 4.0-12.0 & 0.0219* \\
MCH & 16.59±0.67 & 10.81-24.51 & 15.9±0.7 & 13.96-20.64 & 11.0-17.0 & 0.597 \\
MCHC & 33.73±0.81 & 25.5-43.33 & 34.01±0.69 & 31.66-38.75 & 30.0-36.0 & 0.850 \\
\hline
\end{tabular}
\textsuperscript{a} Mean± SE; *Infected and healthy cattle differ significantly at <0.05; **significant at <0.001.Hb: Haemoglobin, PCV: packed cell volume, TEC: total erythrocyte count, MCV: mean corpuscular volume, MCH: mean corpuscular haemoglobin, MCHC: mean corpuscular haemoglobin concentration, TLC: total leukocyte count. Ω: Reference range adopted from Jain, 1993.}
\end{table}

Fig 2: Detection of \textit{Babesia bigemina} from whole blood samples using direct blood PCR method. M: 100 bp DNA ladder; Lane 1: Negative control; Lane 2-8: samples with various degree of parasitaemia.
Table 2: Mean values* of biochemical parameters of crossbred cattle infected with *Babesia bigemina*

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Infected Cattle (n=30)</th>
<th>Healthy control (n=10)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean± SEM</td>
<td>Range</td>
<td>Mean± SEM</td>
</tr>
<tr>
<td>Ca (mg/dL)</td>
<td>9.20±0.13</td>
<td>8.1-11.2</td>
<td>9.64±0.16</td>
</tr>
<tr>
<td>P (mg/dL)</td>
<td>4.99±0.11</td>
<td>3.9-6.0</td>
<td>4.58±0.14</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>78.83±8.95**</td>
<td>13-199</td>
<td>33.3±2.36</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>146.13±7.62**</td>
<td>85-299</td>
<td>80±2.28</td>
</tr>
<tr>
<td>Glucose (gm/dL)</td>
<td>68.95±3.71</td>
<td>NS</td>
<td>56.1±1.71</td>
</tr>
<tr>
<td>TSP (g/dL)</td>
<td>6.12±0.13NS</td>
<td>4.3-7.1</td>
<td>6.9±0.20</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>2.39±0.09**</td>
<td>1-3.4</td>
<td>3.19±0.062</td>
</tr>
<tr>
<td>Globulin (g/dL)</td>
<td>3.73±0.16</td>
<td>1.9-5.8</td>
<td>3.71±0.22</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>27.09±1.02**</td>
<td>20.3-42.4</td>
<td>19.63±1.06</td>
</tr>
<tr>
<td>Cr (mg/dL)</td>
<td>1.93±0.1**</td>
<td>1.3-3.9</td>
<td>1.03±0.18</td>
</tr>
<tr>
<td>TBIL (mg/dL)</td>
<td>1.42±0.06**</td>
<td>1.2-2.2</td>
<td>0.72±0.12</td>
</tr>
<tr>
<td>DBIL (mg/dL)</td>
<td>0.41±0.05NS</td>
<td>0.1-1.0</td>
<td>0.23±0.03</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>57.3±4.21NS</td>
<td>34-96</td>
<td>48.6±3.17</td>
</tr>
<tr>
<td>Chol (mg/dL)</td>
<td>113.26±6.26NS</td>
<td>52-175</td>
<td>131.3±4.25</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>32.56±2.94**</td>
<td>37-55</td>
<td>45.9±2.53</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>69.24±6.47**</td>
<td>20-143</td>
<td>75.68±5.98</td>
</tr>
</tbody>
</table>

*Mean± SE; *Infected and healthy cattle differ significantly at <0.05; **significant at <0.001.


and biochemical parameters of *B. bigemina* infected crossbred cows may be useful in understanding the disease pathogenesis, undertaking appropriate corrective measures and effective treatment.

CONCLUSION

Babesiosis, caused by *Babesia bigemina*, adversely affects liver and kidney of the affected animals which is also evidenced in the present investigation through disturbance in serum protein fractions, hepatic and renal dysfunction. For epidemiological investigations, standardized blood direct PCR assay may suitably be utilized for detecting extremely low parasitemia in carrier animals/subclinical infections.

ACKNOWLEDGEMENTS

The authors express their sincere sense of gratitude to the Vice Chancellor, LUVAS, Hisar for providing research facilities and financial support.

REFERENCES


