Molecular Characterization and Phylogenetic Analysis of Babesia Species Isolated from Domestic Cattle

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ABSTRACT
Babesiosis is a key tick born disease caused by the protozoal parasites belonging to the genus Babesia, cosmopolitan in nature and infecting enormous range of large ruminants. Study was intended to determine the presence of local isolates of Babesia species (B. bovis, B. bigemina) their molecular characterization and phylogenetic relationship in cattle (district Narowal) Punjab. Blood samples were collected from 200 suspected cattle, from seven villages of selected district. DNA was extracted by using DNA zole, Gene-all Kit, manual method and subjected to PCR for amplification using specific RLB and Universal Primers. Quantification of protozoan parasite was performed by RT PCR using Solis biodine kit. A total of 36 out of 200 animal samples, were found positive by microscopy. PCR positive samples (17) were amplified and bands of strength 520-bp and 800-bp with specific RLB and for universal primers respectively were obtained for Babesia species. These 17 samples were sent for sequencing and by using Clustal W; Bioedit software with Neighbor-joining method phylogenetic tree was established. Current study reported the presence of B. bigemina i.e. 18% (conventional method) in large ruminants of district Narowal, Pakistan, and confirmation by molecular characterization PCR (8.5%) and phylogenetic relationship. Phylogenetic results showed that our study has homology with Babesia bigemina strain.

INTRODUCTION
The major constraints of livestock industry are tick borne diseases. In tick transmitted diseases, the imperative one is the tick fever, caused by intra-erythrocytic parasite of genus Babesia (Apicomplexa: piroplasmadia: babesidiae). Tick fever is most ubiquitous disease in its nature. Substantially backlog like reduced reproductive fertility and even deaths observed due to these tick borne diseases in livestock population (Vannier and Krause, 2009). Babesia after Trypanosome is the second most dangerous and prevalent haemo-parasite for mammalian host (Barandika et al., 2006). Babesia species are classified according to their hosts, i.e. Cattle (B. bovis, B. bigemina, B. divergens, B. major) Buffalo (B. bovis and B. bigemina) etc. (Uilenberg, 2006). High temperature and anemia are the common clinical signs of babesiosis (Chisu et al., 2019). Hemoglobinuria is a typical sign not commonly observed in other protozoan diseases. Other signs include anorexia, yellow mucous membrane, nasal and ocular discharge and reduction in milk yield in lactating animals (Muraldeedharan, 2015). About 500 million cattle are at risk throughout the globe (Chaudhry et al., 2010; Oscar and Cristian 2018). Tick borne diseases cause 18.6 billion losses and deficiency of 3 billion pieces of hide in cattle per year (Terkawi et al., 2012). Economic losses of production by ticks are about 13-19 billion per year globally (De Castro and Newson, 1993; Oscar and Cristian, 2018). Diminazine aceturate and imidocarb dipropionate are used in the treatment of Babesiosis (Mosqueda et al., 2012).

Focus on the mitochondrial Cytochrome c Oxidase Subunit I (COXI) gene in the present study was due to its nature of being the most conservative protein-coding
genes in the mitochondrial genome of animals. COXI exhibit fast mutation rate and easily distinguished between closely related species (Hong et al., 2019), preferred for the evolutionary time depths. Internal Transcribed Spacer primers reveal highest degree of variation along its genomic sequences via unequal crossing over and gene conversion (Eui et al., 2016).

The molecular characterization and phylogenetic analysis was opted as such for the confirmation of parasites as by (Oliveira-Sequeira et al., 2005; Rajabi et al., 2017; Barbara et al., 2017; Jafarbeklo et al., 2018; Nast et al., 2018; Pardeep et al., 2019 ). For prevention and immunization of calves, vaccines are used mostly imported ones. It is imperative to get idea about the prevalent strain and their genetic evolutionary relationship to be taken as candidate vaccinal antigen (local isolates). The purpose of present study is to know the genetic makeup of prevalent strains of Babesia with parasite of other areas to estimate the extent of disease caused by different strains.

**MATERIALS AND METHODS**

**Sampling and identification:** A total number of 200 blood samples were randomly collected from suspected cattle in district of Narowal. Sampling was performed on the basis of number of animals present in each station. Blood was collected from jugular vein into EDTA-coated vacutainers and transported. Staining was performed by thin and thick blood smear.

**DNA Extraction and PCR:** The preliminary screened samples were subjected to DNA extraction. For comparative study DNA extraction was performed by three different methods (DNAzol BD, Kit & Manual method) as per manufacturer’s instructions.

**Kit method:** GeneAll Exgene kit was used for the purification of total DNA from blood samples. DNA was extracted by using kit by following the directions.

**Manual method:** DNA was extracted by manual method by using lysis buffer, TEN buffer and PCI buffer. The concentration of DNA was estimated by using the Nano Drop (Mtshali et al., 2013).

Two sets of primers were selected for the amplification of DNA sample. One set of primers 18s ribosomal DNA specific for Babesia species termed as RLB primers of product size 520-bp was used for amplification. Second set of primers was universal (COXI) i.e. 1202 and 400 primers which amplified a product of 800-bp size (Table 1).

**PCR and conditions:** To confirm the B. bigemina and B. bovis PCR was executed by using 25-μl reaction mixture, having 2μl of extracted DNA, 2.5μM of each primer and 15μl of Dream Taq Green PCR Master Mix. The PCR reactions were performed in an automated Thermal Cycler (Bio Rad, USA). Each cycle consisted of denaturation @ 94°C for 1 min., an annealing step of 1 min. at 55°C (with a bit modification) followed by 40 cycles and extension @ 72°C for 1.5 min. Preceding’s the final extension step at 72°C for 10 min. (Gubbles et al., 1999).

The condition for universal primer was initial denaturation consist of 96°C for 10 minute followed by 94°C for 30 second annealing at 48°C for 30 second followed by 35 cycles extension at 72°C for 90 second and final extension at 72°C for 10 minute. Agarose gel 1.5% stained with Biotium GelRed Acid Stain was used to analyze the PCR-generated amplicons by electrophoresis. The gel electrophoresis was operated at 113 Volts for 35 minutes. DNA Ladder was also used as Molecular weight marker (Mtshali et al., 2013). A 100 kb molecular weight marker was run along with samples.

**Sequencing:** The confirmed PCR product was sent to laboratory Advance Bioscience International First base Malaysia for sequencing. Nucleotide sequences were calibrated and arranged by using Bioedit software. The consensus sequences which were obtained by using Bioedit software, was trimmed manually to the equivalent length for the construction of phylogenetic tree. The other software Mega-7 and neighbor-joining methods was used for the construction of phylogenetic tree (Criado-Fornelio et al., 2003). The Basic Local Alignment of BLAST Search Tool was used to check the sequence similarity between our data and in database. Sequence homology helped to identify the putative gene. Sequences were checked on NCBI website by using URL Genbank. http://www.ncbi.nlm.nih.gov/nuccore.

**RT- PCR:** The Solis biodine kit was used for Real time PCR. Each cycle consisted of denaturation @ 94°C for 1 min., an annealing step of 1 min. at 55°C (with a bit modification) followed by 40 cycles and extension @ 72°C for 1.5 min. Preceding’s the final extension step at 72°C for 10 min. The results for quantification of parasites were recorded in terms CT values.

**Statistical analysis:** The data pertaining prevalence was imperiled to chi-square test and univariate analysis for analyses using Statistical Package for Social Sciences (SPSS) version 20. P-value less than 0.05 were considered significant.

**RESULTS**

Samples of cattle (200 in number) were processed for the identification of Babesia under microscope after staining procedure. Thin blood smear was stained with Giemsa and observed under oil immersion lens of microscope i.e. 40x. Intra erythrocytic bodies were found in heavy concentration with Giemsa Stain. Blood sample was also stained with field stain B and intra-erythrocytic bodies were observed. These intra-erythrocytic bodies were found inside the red blood cells and these were horse shoe shape.

Out of 200 samples 36 were found positive i.e. they have intra erythrocytic bodies. Percentage of infection was 18% by microscopic examination. The PCR detected 17 samples positive (Table 2). PCR reactions targeting the 18S ribosomal DNA (RLB-Internal Transcribed Spacer Primers) of Babesia bigemina species was tested for its ability to amplify the target Babesia species with 100bp ladder. Exact 520 bp amplified product was retrieved. The amplified DNA used (Fig. 1) in PCR was of Babesia.
Fig. 1: PCR reactions targeting the RLB Primers of Babesia bigemina species that infect cattle & buffalo. Primer pair was tested for its ability to amplify the target Babesia species with 100bp ladder in lane #1. Lane #2 Positive Control, Lane #3 Negative Control, Lane #4, 5 & 7 Samples. Exact 520 bp amplified product was found.

Fig. 2: PCR reactions targeting the Mitochondrial Cytochrome c Oxidase subunit I (COI) loci of Babesia bigemina species that infect cattle & buffalo. Primer pair was tested for its ability to amplify the target Babesia species with 100bp ladder in Lane #1. Lane # 2, 3, 4 & 5 samples. Exact 800 bp amplified product was instituted.

Fig. 3: Partial sequence of Babesia species WA-1 isolate from Narowal (18S Ribosomal RNA gene)

Fig. 4: Phylogenetic analyses sequences obtained from PCR positive sample. The Phylogenetic Tree reflecting the exact position of Babesia bigemina present the District Narowal, Pakistan.
The Mitochondrial Cytochrome c Oxidase subunit I (COI) loci with universal Primers (1202 Reverse and 400 Forward) of Babesia bigemina species that infect cattle was experienced to amplify the target Babesia species. Precise 800 bp amplified product was obtained (Fig. 2).

**Sequencing and phylogenetic:** The tree (Fig. 4) showed a similar ancestor relationship of Babesia species with other areas as found in district Narowal. The sequences of the present study reflected to be distributed in same cluster of Babesia bigemina as well as same clade. But the constructed phylogenetic tree revealed that one sequence (B. bigemina Nar/Pak-8 Fig. 3) clustered in a different sub clade, denoted to be genetically different from other isolates collected from Narowal.

Disparity index per sites in comparison with area of present study for all sequences is presented in Table 3. The Zero value reflected the no difference while the values greater than zero indicate the higher difference in base composition. Evolutionary analyses were conducted in MsEGA7.

**RT-PCR:** The Ct value for Babesia bigemina was calculated by real time PCR and the samples showed fluorescence of sample R (purple) 22.6 and sample RM (yellow) 22.14 (Fig. 5). The NTC was used as threshold and two samples were amplified; named as R and Rm. AT cycle no. 20 the reaction shows fluorescences. This shows that it starts amplification at cycle no. 20 and the maximum shows at ct value 22.55 and 22.14.

**DISCUSSION**

Tick borne diseases impose considerable constraints on cattle and buffalo health and economic development in temperate areas of the world (Barandika et al., 2006). Annually 500 million dollars economic losses are observed because of tick fever and most losses occur due to B. bovis infection (Ramos et al., 2012). These samples were screened for the phylogenetic analysis of B. bovis and B. bigemina. Although the distribution of B. bovis and B. bigemina was found all over the country but a notable observation was made during the screening of blood samples of district Narowal i.e. the distribution of B. bigemina was more than 18% i.e. in 200 suspected blood samples 36 were found as positive by microscopy which is in coherence with a previous study in which prevalence rate of B. bigemina was 20.66%. The high prevalence rate was found in low age group and in females (Saad et al., 2015; Khan et al., 2016).

Another study showed a high prevalence rate of B. bigemina in which 24 samples were found positive out of 100 for B. bovis and B. bigemina by microscopy (Saad-Roy et al., 2015) which is in accordance with present study. The prevalence rate of B. bigemina around 19% and of B. bovis was 11% by Ahmad et al. (2014) is also in agreement with current findings. The prevalence rate in calves less than one year was higher than aged calves. As for as sex wise prevalence was concerned, female calves were more prone to Babesia than male calves (Ahmad et al., 2014; Abderasol et al., 2017).

Out of 36 microscopy samples only 17 samples got amplified. PCR reactions targeting the 185 ribosomal DNA of Babesia bigemina species was tested and exact 520 bp amplified product was retrieved which is in coherence with Gubbles et al. (1999).

The Mitochondrial Cytochrome c Oxidase subunit I (COX1) loci with universal Primers of Babesia bigemina species was experienced and 800 bp amplified product was instituted as that in case of Ogedebge et al. (2011) and Hong et al. (2019).

**Table 1:** Two sets of primers were selected for the amplification of DNA sample. One set of primers 18s ribosomal DNA specific for Babesia species (RLB primers). Second set of primers was universal (COX1) i.e. 1202 and 400

| Specie       | Primer name          | Primer sequence                        | Target region | Product size |
|--------------|----------------------|----------------------------------------|---------------|--------------|
| Babesia      | RLB(Forward)         | 5'-GAGTATG3AACAAGAATAAACAAATA-3'        | 18s ribosomal | 520bp        |
| Babesia      | RLB(Reverse)         | 5'-TCTTCGACTCCCTAACTTTC-3'             |               |              |
| Babesia      | 400(Forward)         | 5'-GGTTCAGTTGTTGAGAGG-3'               | COX1 specific | 800bp        |
| Babesia      | 1202(Reverse)        | 5'-CCAAKRAYHGCACCAAGAGATA-3'           |               |              |

(Ogedenbge et al., 2011; (Gubbels et al., 1999).

**Table 2:** Percentage of Positive and Negative Samples by Micrometry & Molecular characterization of Babesia bigemina (Chi square)

| Microscopy | PCR |
|------------|-----|
| Positive   | 36  |
| Negative   | 17  |
| Total      | 200 | 200 |

P value=9.13E-060.000009 (Chi square was used to get the percentage).

**Table 3:** Estimates of net base composition bias disparity between sequences of babesia species prevalent in narowal and other areas of Pakistan

| SS2 RLB   | SS3 RLB | SS4 RLB | SS7 RLB | Ikhlas, Darman, Bara, | Nagoo, Darman, Bara, | Narowal, Darman, Bara, |
|-----------|---------|---------|---------|-----------------------|----------------------|------------------------|
| 0.000     | 0.000   | 0.000   | 0.000   |                       |                      |                        |
| 0.000     | 0.000   | 0.000   | 0.000   |                       |                      |                        |
| 0.000     | 0.000   | 0.000   | 0.000   |                       |                      |                        |
| 0.000     | 0.000   | 0.000   | 0.000   |                       |                      |                        |
| 0.000     | 0.000   | 0.000   | 0.000   |                       |                      |                        |
| 0.000     | 0.000   | 0.000   | 0.000   |                       |                      |                        |
| 0.000     | 0.000   | 0.000   | 0.000   |                       |                      |                        |
| 0.000     | 0.000   | 0.000   | 0.000   |                       |                      |                        |

Fig. 5: Graphical representation of Real time PCR for Babesia bigemina as of X-axis number of cycles and on Y-axis Fluorescence. The Ct value for Babesia bigemina samples showed fluorescence of sample R (purple) 22.6 and sample RM (yellow) 22.14.
Phylogenetic results showed that our study has homology with B. bigemina strain.

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