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Molecular Characterization and Assessment of Hematobiochemical and Oxidative Indices in Dog Naturally Infected with *Babesia canis vogeli*

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ABSTRACT

Background: The study was aimed at molecular detection and assessment of important biomarkers in the natural cases of canine babesiosis.

Methods: Blood samples of 239 dogs were examined in PCR by targeting 18S rRNA gene. Hematobiochemical, oxidant-antioxidant and plasma cortisol parameters were estimated in the dogs on the day of presentation.

Result: The 18S rRNA gene sequence showed 100% homology with *Babesia canis vogeli* and phylogram formed a tight cluster of *B. canis vogeli* originated from India/other countries. Higher prevalence rate (P<0.05) was noted in the PCR (7.95%) than the cytological technique (3.76%). Hemogram of infected dogs showed decrease (P<0.05) in the mean value of hemoglobin, RBC, WBC, HCT, whereas an increase in MCHC, lymphocytes, eosinophils, monocytes and thrombocytes. The ALT (49.29±1.53 U/L), AST (48.33±2.93 U/L), total protein (10.56±0.60 g/dL), creatinine (1.41±0.10 mg/dL) and urea (19.32±0.97 mg/dL) showed significant (P<0.005) increase, whereas decrease in the levels of serum glucose (82.76±2.78 mg/dL) in the infected dogs. Activity of MDA and SOD was significantly (P<0.01) increased (7.50±7.08 nmole/µL blood) and decreased (0.015±3.91 nmole/µL blood) in the diseased dogs, respectively. Plasma cortisol concentration was 11.10±7.84 nmol/L and 2.77±5.78 nmol/L (P<0.01) in the infected and uninfected dogs, respectively.

Key words: 18S rRNA, Anemia, Cortisol, Gene, MDA, PCR.

INTRODUCTION

Amongst the common tick-borne diseases of canidae, babesiosis is one of the major concerns in tropical countries including India. It is caused by intraerythrocytic apicomplexan protozoa of *Babesia* genus. *Babesia canis* occurs as a single pear shaped piroplasm and *B. gibsoni* often exists in individual ring forms. The large form is genetically characterized into *B. canis canis*, *B. canis vogeli* and *B. canis rossi* (Carret *et al*., 1999).

The destruction of parasitized or non-parasitized erythrocytes causes mild to severe form of anemia (Köster *et al*., 2015). Along with abnormal hematological parameters, there can be hypoglycaemia, increased serum activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum creatinine and serum urea (Brahma *et al*., 2019). Oxidative damage to the erythrocytes may contribute to the pathogenesis of anemia in babesiosis (Crnogaj *et al*., 2010). Malondialdehyde (MDA) is an end product of oxygenation of polyunsaturated fatty acids, thus commonly used as oxidant biomarker for assessing oxidative stress in the cells or tissues. There are several enzyme systems that catalyze reactions to neutralize the reactive oxygen species in the cells. Few of them are superoxide dismutase (SOD) and glutathione reductase (GR)/ glutathione disulfide reductase (GSR), which can be used as an antioxidant biomarker to evaluate the oxidative stress in canine babesiosis (Crnogaj *et al*., 2017). The constant stress in the *Babesia* infected dog causes elevated levels of basal and post-adrenocorticotropic hormone stimulated serum cortisol concentrations (Schoeman and Herrtage, 2008).

Nevertheless, it warrants a rapid and accurate diagnosis for instituting appropriate therapy and control in dogs in the endemic areas. Nucleic acid-based detection assays like PCR and its variants are the most promising diagnostic tools with high level of sensitivity and specificity (Kushwaha *et al*., 2018). One of the important gene targets for *Babesia* detection by PCR is 18S rRNA gene (Passos *et al*., 2005).

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It has highly conserved flanking regions, allowing the use of universal primers. Its repetitive arrangement within the genome provides excessive amounts of DNA for PCR (Hillis and Dixon, 1991).

Taking all the above facts into consideration, present study was aimed to identify the causative agent of canine babesiosis in South Gujarat, India using PCR and analyzing the phylogeny based on 18S rRNA gene. The level of hematobiochemical parameters, MDA (oxidant biomarker), SOD and GR/GSR (antioxidant biomarker) and plasma cortisol were also evaluated in the dogs on the day of presentation.

MATERIALS AND METHODS

Animal/area under study

Study was conducted on dogs presented before clinician for the diseases diagnosis and treatment especially with pyrexia, anaemia and tick on body in South Gujarat, India. The work was done at Veterinary College, NAU, Navsari during 2018-2019. Fifteen PCR positive and negative dogs with canine babesiosis were selected as infected and uninfected group, respectively.

Blood collection

Three ml blood was collected per dog in aseptic condition, 2 ml transferred in the sterile vial with K3 EDTA and 1 ml was transferred in a plain tube for plasma/serum separation.

Cytological examination

Ear tip of the dog was pricked to prepare a peripheral blood smear. Giemsa-stained smear was examined for the piroplasm of *Babesia* under oil immersion lens.

PCR examination

Genomic DNA was isolated from 100 ìl of the whole blood using DNEasy Blood and Tissue kit (Qiagen, Germany) as per manufacturer protocol. The eluted DNA of OD of 260 nm: 280 nm of 1.7-1.9 was stored at -20°C. The PCR was performed to amplify the 340 bp of 18S rRNA gene using *Babesia* genus specific primers pair of 5′-gtcttgtaattgg aatgatggtgac-3′ and 5′-atgcccccaaccgttcctatta-3′ (Birkenheuer *et al*., 2003). Reaction mixture of 25 µl contain 0.5-1 µg of template DNA, 1 µl (10-15 pmol) of each primer, 12.5 µl of 2x PCR master mix (Qiagen, Germany) and nuclease free water. Amplification condition was initial denaturation 94°C for 5 min, denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min, 40 cycles and final extension at 72°C for 10 min. Each time the reaction was run with positive/negative/reagent control. All PCR products were electrophoresed through 2% agarose gel containing ethidium bromide and visualized under UV fluorescence.

Gene sequencing and phylogenetic analysis

The DNA was eluted from the agarose gel using a gel extraction kit (Qiagen, Germany) and nucleotide sequence was determined at the Sequencing Department, Eurofins Genomics, Bengaluru. Sequence data was analysed in GeneTool 1.0 Lite. The 5′ and 3′ ends of the 18S rRNA gene sequence were initially determined by comparing them with previously published sequences. Sequences were put to further analysis including similarity search in the BLASTN program of NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). Nucleotide sequence of 100% homology was submitted into the NCBI gene bank. Phylogenetic tree was constructed in Neighbor-Joining (NJ) and Maximum Likelihood (ML) method in the MEGA 10 of version 10.1.7. Bootstrap consensus was inferred from 1000 replicates. Nucleotide sequences were of *B. gibsoni*, *B. canis vogeli*, *B. canis canis*, *B. canis rossi* and *B. microti* (as out group).

Hemogram and hemato-biochemistry

One ml of fresh whole blood was analyzed in blood auto-analyzer (Nihon Kohden, Japan) to ascertain the blood parameters. The serum concentration of glucose, total protein, hepatic enzymes, urea and creatinine were estimated using commercial kits (Randox Laboratory, UK).

Oxidant-antioxidant and plasma cortisol estimation

The RBC pellet was prepared from fresh blood. It is diluted in ice cold distilled water in 1:10 ratio to make 10% hemolysate. Hemolysate was used in Sigma Aldrich assay kit to measure MDA, SOD and GR. Cortisol level was estimated using Immunotag™ Cortisol ELISA Kit (G-Biosciences, USA).

Statistical analysis

Statistical analyses were performed using R software, comparing means using one-way ANOVA with Duncan's multiple range test. P value <0.05 or <0.01 was considered significant.

RESULTS AND DISCUSSION

Giemsa-stained thin blood smear showed *Babesia canis* in blood plasma (Fig 1a) and inside RBC (Fig 1b). Intra-erythrocytic form many a time had multiplied infection of the *Babesia canis* parasites (Fig 1b). Out of 239 screened dogs 9 (3.76%) was infected with babesiosis in cytological examination. The PCR had produced a band of 18S rRNA gene of 340 bp (Fig 2). Current nucleotide sequences (South Gujarat isolate) (MW093397 and MW093398) showed 100% homology and 534-538 total score with published 18S rRNA DNA sequences of *B. canis vogeli* of Asia (KU321851.1) and also with the isolates of Europe, Africa and South America. The PCR had detected 10 more cases (=19 dogs) (7.95%) of canine babesiosis than the cytological examination (P<0.05). Sensitivity and specificity of the PCR was 100% as compared to blood smear examinations in detecting the infection. This finding was in accordance with the finding of researchers in South India (Jain *et al*., 2018) and North India (Laha *et al*., 2014). Malaysian scientist, Prakash *et al*. (2018) used the same gene in the PCR and successfully detected *B. canis vogeli* in ticks (1.4%) and dogs (2.1%).

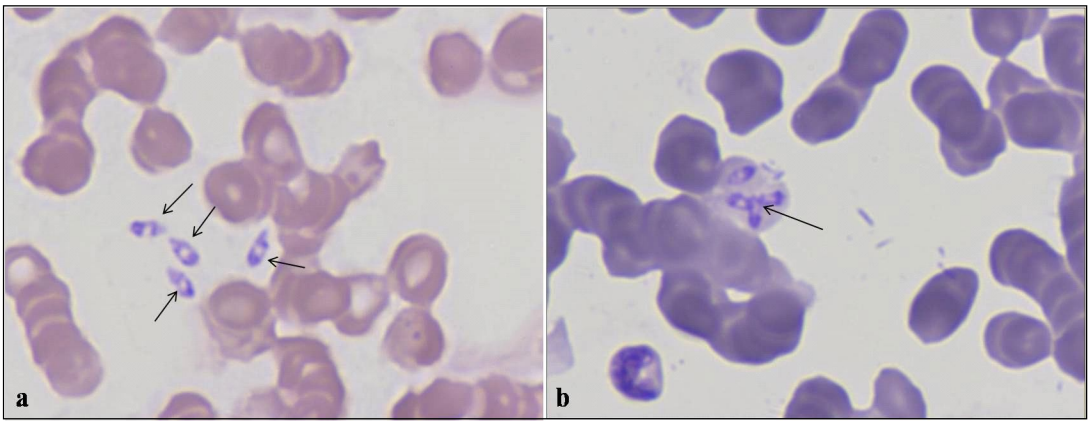

Fig 1: Giemsa stained thin blood smear of *Babesia canis* (black arrow) infected dog.

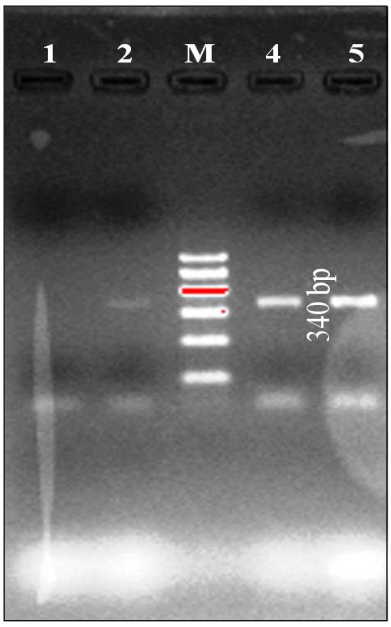

Fig 2: Agarose gel of 2% showing resolved PCR product [Lane 1- No band (negative control); Lane 2 (positive control) and Lane 4-5 (infected)- DNA band of 340 bp; Lane M- DNA ladder].

Topology of the 18S rRNA DNA tree of by distance and character-based methods were almost similar with a small difference in bootstrap values (Fig 3). The current sequence had formed a tight cluster with the *B. canis vogeli* of from different locations (Fig 3). Monophyletic lineage relationships were observed with high bootstrap proportion (98% in NJ and 99% in ML) for current and most of the published nucleotide sequences of *B. canis vogeli*. South Gujarat isolate revealed the closest similarity with Kerala, India isolates and also with the isolates reported from other countries of Asia, Australia and South America (Fig 3). Furthermore, the phylogram revealed separate clades of *B. canis vogeli*, *B. canis canis*, *B. canis rossi* and *B. gibsoni* (Fig 3). The same gene was used by Passos *et al*. (2005) and Solano-Gallego *et al*. (2008) to confirm the incidence of *B. canis vogeli* in Brazilian and Italian dogs, respectively.

Hemogram showed mild to moderate anemia and erythropenia in most of the infected dogs (Table 1). Infected dog noted decreased HCT value as compared to *Babesia* free dogs (P<0.05). A low level of RBC count, hematocrit and hemoglobin are an indication of anemia in the infected dogs (Varshney *et al*., 2003). Anemia might be due to direct mechanical disruption caused by the parasite, along with extra/intra vascular hemolysis and immune/non-immune mediated destruction of RBCs (Salem and Farag, 2014). There was non-significant difference in MCV and MCH value between the two group (normocytic normochromic anemia), but MCHC observed significant decrease in the infected animals (hypochromic microcytic anemia) (Table 1) while Salem and Farag (2014) observed non-significant difference in these parameters. Mean WBC count in infected dogs observed marked (P=0.004) leukocytosis with lymphocytosis and monocytosis. However, there was non-significant (P>0.05) increase in the granulocyte count in the infected dogs. Eosinophilia (P=0.000) and thrombocytosis (P<0.01) were also evident in the infected dogs. There was a corresponding increase in the MPV (P<0.01) and PDW (P>0.05) in the infected than the uninfected dogs. Increased value of RDW% in *Babesia* infected dogs was due to the formation of large size reticulocytes and young RBCs than mature erythrocytes (regenerative anemia) (Fabisiak *et al*., 2010).

Plasma biochemical profile of infected dogs revealed significantly (P<0.01) decreased values of blood glucose, and it was in agreement with Keller *et al*. (2004) (Table 1). Hypoglycemia can be due to anorexia and impaired hepatic function. Increased level of total protein has (P<0.01) been a most frequently reported feature of canine babesiosis (Hossain *et al*., 2003). Elevated level of ALT and AST (P<0.01) in the infected dogs was in agreement with previous observation of Furlanello *et al*. (2005) and it is an indication of hepatic tissue damage. Creatinine level in the infected dogs was significantly higher than the uninfected group. A similar observation was noted by Gonde *et al*. (2017) but Konto *et al*. (2014) observed lower levels of creatinine in *Babesia* infected dogs. Increased level of BUN and

creatinine were the indication of renal degenerative changes during canine babesiosis (Brahma *et al*., 2019). Consequently, an increase ($p<0.05$) in the level of serum urea was observed in the infected as compared with the uninfected dogs in the current (Table 1) and Lobetti *et al*. (2012) study.

In the infected dogs, the activity of MDA and SOD was significantly ($P<0.01$) increased and decreased, respectively (Table 1). Significantly an increased level of MDA was noted in dogs infected with *B. canis canis* (Crnogaj *et al*., 2010, 2017). A significantly reduced activity of SOD ($P<0.01$) in

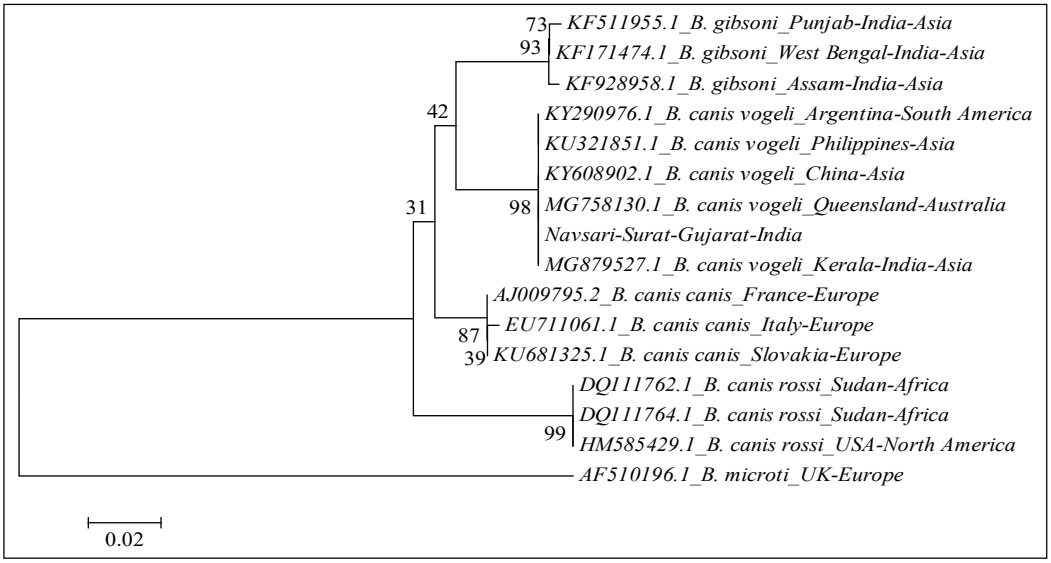

Fig 3: Phylogram of current and the published nucleotide sequences of 18S rRNA constructed using Neighbor-Joining method.

Table 1: Hematology, biochemical analysis and oxidant anti-oxidant status of dogs on day of presentation (Mean±SE).

Parameters		Infected group	Uninfected group	P value
Hematology	Hb (gm/dL)	9.35^{a}±0.38 (4.9-14.01)	15.45^{b}±0.61 (11.1-19.1)	0.000
	RBC (10^6/µL)	4.50^{a}±0.15 (2.29-5.94)	6.60^{b}±0.38 (4.59-8.35)	0.000
	HCT (%)	35.75^{a}±1.52 (15-47.4)	43.72^{b}±0.44 (30.1-54.6)	0.016
	MCV (fL)	67.17±0.62 (60.29-70.61)	66.37±0.30 (61.5-72.9)	0.888
	MCH (Pg)	22.08±0.31 (18.32-25.64)	23.43±0.28 (21.9-25.7)	0.888
	MCHC (gm/dL)	33.26^{a}±0.56 (24.61-36.1)	35.37^{b}±1.90 (34.1-36.8)	0.041
	RDW (%)	14.02±0.13 (12.35-15.65)	11.69±0.06 (10.9-13.7)	0.126
	WBC (10^3/µL)	13.93^{a}±0.57 (4.55-20.5)	10.05^{b}±0.25 (4.2-12.9)	0.004
	Lymphocyte (10^3/µL)	4.97^{a}±1.73 (3.36-6.66)	2.40^{b}±0.14 (1.59-2.91)	0.000
	Monocyte (10^3/µL)	1.15^{a}±0.08 (0.45-241)	0.62^{b}±0.19 (0.12-1.2)	0.022
	Granulocyte (%)	74.43±1.62 (54.1-88.8)	68.97±0.23 (61.6-78.7)	0.170
	Eosinophil (%)	4.79^{a}±0.44 (0.90-10.24)	1.40^{b}± 0.57 (0.4-2.3)	0.000
	Thrombocytes (10^3/µL)	306.53^{a}±6.35 (220-357)	210.60^{b}±0.54 (132-284)	0.000
	MPV (fL)	13.05^{a}±0.58 (5.9-18.27)	8.07^{b}±0.60 (5.64-10.42)	0.000
	PDW (%)	64.27±092 (57.65-80.21)	61.63±0.35 (53.2-67.34)	0.184
	PCT (%)	0.18±0.02 (0.05-0.38)	0.15±0.29 (0.08-0.3)	0.126
Biochemical analysis	Blood glucose (mg/dL)	82.76^{a}±2.78 (65.09-121)	108.13^{b}±1.34 (100-116)	0.000
	Total protein (g/dL)	10.56^{a}±0.60 (4.77-18.87)	5.59^{b}±0.30 (4.01-7.22)	0.000
	Serum creatinine (mg/dL)	1.41^{a}±0.10 (0.39-1.98)	0.74^{b}±0.02 (0.58-0.9)	0.001
	Serum urea (mg/dL)	19.32^{a}±0.97 (11.32-34.09)	14.90^{b}±0.89 (8-19.9)	0.023
	ALT (U/L)	49.29^{a}±1.53 (40.4-62.12)	18.04^{b}±0.98 (12.2-26.98)	0.000
	AST (U/L)	48.33^{a}±2.93 (31.26-86.31)	35.79^{b}±0.82 (31.2-40.98)	0.000
Oxi- or antioxidant	MDA (nmole/µL)	7.50^{a}±7.08 (0.078-6.818)	2.24^{b}±3.87 (2.35-14.36)	0.001
	SOD (U/mL)	0.015^{a}±3.91 (0.08-0.21)	0.244^{b}±3.90 (0.19-0.29)	0.000
	GR (U/mL)	1.06±4.19 (0.83-1.79)	1.86±4.10 (1.121-2.39)	0.079

Note: - Values with superscript a, b differs significantly in a row. Values in parentheses indicate range.

the *B. canis vogeli* infected dogs was noted, this result agrees with the finding of Crnogaj *et al*. (2017) in *B. canis canis* infected dogs. A non-significant reduction in the amount of GR was observed in *B. canis vogeli* infected dogs. Overall, a low amount of antioxidant biomarkers in *B. canis vogeli* infected dogs, can be due to the consumption of these biomolecules in neutralizing the free radicals produced during infection. Significantly ($P<0.01$) higher mean cortisol concentration was noted in the *B. canis vogeli* infected dogs of current study which agree with the finding of Schoeman *et al*. (2007) in *B. canis rossi* infected dogs.

CONCLUSION

Babesia canis vogeli was found to be the dominant organism for canine babesiosis in South Gujarat, India, as evident in 18S rRNA gene-PCR and NCBI-BLAST study. Phylogram of 18S rRNA gene formed a tight cluster of *B. canis vogeli* originated from India and other parts of the world. Infected dogs measured significantly decreased value of mean hemoglobin concentration, RBC count, blood glucose and SOD, whereas significantly increased values of total protein, serum creatinine, ALT, AST and MDA as compared to the uninfected dogs. Oxidant-antioxidant imbalance in the infected dogs support the notion that infection is associated with oxidative stress and its course may be linked to the resultant anemia.

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