

A STUDY ON BOVINE BABESIOSIS AND TREATMENT WITH REFERENCE TO HEMATO-BIOCHEMICAL AND MOLECULAR DIAGNOSIS

Mohamed A. Hashem¹, Ahmed N.F. Neamat-Allah^{1*}, Mohamed A. Gheith²

¹Department of Clinical Pathology, Faculty of Veterinary Medicine, Zagazig University,

²Veterinarians at Veterinary Clinic, Dakahlia Governorate, Egypt

*Corresponding author, E-mail: anattia@vet.zu.edu.eg; drnemovete@yahoo.com

Abstract: This study was carried out during the period from May to December 2015 on thirty crossbred female cows in Sherbeen city, Dakahlia Governorate and two to four years of age. Twenty cows suffered from fever, anorexia, increase in respiratory and heart rates, anemia, pale to icteric mucous membranes and red urine. Babesiosis was diagnosed clinically and confirmed by detection of intra-erythrocytic stages of the *Babesia* in Giemsa stained blood film, polymerase chain reaction amplification (PCR) and sequencing of 18S rRNA gene. Hemoparasites were detected in thirteen blood samples by microscopic examination, whereas PCR were positive in twenty. The hematological findings revealed a marked decrease in the erythrocyte count, hematocrit %, hemoglobin concentration and mean corpuscular hemoglobin concentration with a significant increase in mean corpuscular hemoglobin and mean corpuscular volume values in *Babesia*-infected cows when compared with healthy control. On the other hand there was a significant leucopenia and thrombocytopenia along with a significant eosinophilia. The biochemical findings of infected cows revealed a significant increase in activities of serum alanine aminotransferase, aspartate aminotransferases, alkaline phosphatase and lactate dehydrogenase. In contrast a significant decrease in serum levels of total proteins, albumin, globulins, sodium and potassium. While serum bilirubin (total, direct and indirect), urea and creatinine levels were significantly increased. After administration of single I/M imidocarb dipropionate 12% (1.7 mg/kg BW) to *Babesia* infected cows, there was an improvement in hematological and biochemical parameters. It concluded that molecular detection of *B. bigemina* more sensitive than blood smear. Treatment infected cows with imidocarb improves the clinical signs, hematological and biochemical parameters that indicate recovery of infected cows.

Key words: babesiosis; cow; erythrogram; imidocarb; PCR; 18S rRNA; Egypt

Introduction

Babesiosis is a tick borne hemoprotozosis worldwide affecting several species of mammals and caused by multiplication of apicomplexans in the *Babesia* genus intra-

erythrocytes. The large number of species described more than one hundred is an evidence of the evolutionary success of this parasite (1). The most economical tick borne disease of livestock is bovine babesiosis. It spreads in Africa, Australia, Central and South America.

Many species of *Babesia* can cause bovine babesiosis but the most important bovine babesias are *B. bigemina*, *B. bovis* and *B. divergens* (2). The disease is characterized clinically by sudden onset of acute fever, weakness, rumen stasis, anorexia, anemia and an increase in heart and respiratory rates, pale or icteric mucous membranes, in addition to hemoglobinuria (3).

Economic importance for babesiosis are increased due to abortions, reduction of milk or meat production and even mortality, so increase the demand of encounter the tick vector and vertebrate host by acaricide and therapeutics approach respectively (2). Routine classical diagnosis of acute cases of babesiosis is based on the examination of blood smears that is considered the gold standard, while, PCR and gene sequencing allow the diagnosis of faint parasitemia that couldn't be identified by the classical methods (4).

Imidocarb dipropionate is a urea derivative used in veterinary practice as an antiprotozoal agent for the treatment of babesiosis and other hemoprotozoosis (5). Due to the huge losses and increasing trend of babesiosis in cattle, the present study was implemented to diagnose the infected cattle with Babesiosis in the relation to some hematological and biochemical changes occurs during and after drug therapy.

Material and methods

Animals

The present study was implemented during the period from May to December 2015 on thirty crossbred female cows in Sherbeen city, Dakahlia Governorate, aged from two to four years. Ten cows were clinically healthy and confirmed by laboratory means, used as a normal control and twenty cows showed clinical symptoms of babesiosis (fever, pale or icteric mucous membranes and hemoglobinuria) and confirmed by detection of intra-erythrocytic stages of the hemoparasite in stained blood film from ear vein. The diseased cows were treated with Imizol® (Schering-Plough Animal Health, Germany). Each ml contains imidocarb 85mg as 12% imidocarb

dipropionate by a single I/M injection with 1.7 mg / kg BW (6).

Blood Samples

The blood samples (n=30) were gathered from the jugular vein of all animals (control and infected cows) during the feverish period and 7 days post treatment. The samples were separated into two divisions. The 1st was put in chemically free tubes containing anticoagulant (dipotassium salt of EDTA) for hematological and molecular studies, while the 2nd division was collected in centrifuge tubes without anticoagulant for separation of serum. After clotting, the blood samples were centrifuged at 3000 rpm for 15 minutes and the clear serum was carefully aspirated into chemically free and clean tubes and stored at -20°C until used later for biochemical parameters analysis.

Molecular diagnosis of Babesiosis by PCR

Whole blood sample (200µl) was used for extraction of genomic DNA by G-spin™ Total DNA Extraction Kit (iNtRON Biotechnology, Inc., South Korea) following the manufacturer instructions.

The complete nucleotide sequences of 18S rRNA of all piroplasms were downloaded from GenBank and aligned by MAFFT alignment in Geneious software. Consequently, one primer set was designed from a conserved area using Integrated DNA technology online website (<https://eu.idtdna.com/PrimerQuest/Home/Index>). The primer set amplify 733 bp from the 18S rRNA gene of piroplasms in the examined blood samples (n=30). The sequences of the primers are forward Piro-18-F2: 5'-'ACT GTC AGA GGT GAA ATT CTT AG-3'' and reverse Piro-18-R2: 5'- AAT AAT TCA CCG GAT CAC TCG- 3''. The amplification condition was as follow, denaturation initiated at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 63.1°C for 30 sec, extension at 72°C for 30 sec, and final extension at 72°C for 5 min (4). 5 µl of the PCR product was electrophoresed along with 100bp DNA molecular weight 1% agarose gel containing ethidium bromide (at the rate of 0.5µg/ml) at constant 80V for 30 minutes in 1X TAE buffer. To confirm presence of *B.*

bigmina, DNA sequencing of the amplified product of 18S rRNA region (733 bp) was performed in one representative sample. The GenBank accession number obtained was AAA6558. It was performed in an automatic sequencer at Macrogen Corp. (Seoul, South Korea), after purification of amplicons using a PCR purification kit Solgent Co Ltd (South Korea). The sequences were trimmed then analyzed using Geneious bioinformatics software (Biomatters). Consequently, the trimmed sequences were identified in GenBank by search in basic local alignment search tool (BLAST).

Hematological studies

Hematological examination included estimation of RBCs count, Hb concentration, PCV, MCV, MCHC, MCH, platelets, total and differential leukocytic counts by using full automatic digital cell counter (Sysmex XS-800i, Germany).

Biochemical studies

The obtained serum from centrifugation of coagulated blood was used for estimation of alanine and aspartate aminotransferases (ALT and AST) according to Reitman and Frankel (7). Alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) according to Wu (8). Total proteins, albumin, globulins and albumin/globulins ratio (A/G ratio) were measured as previously reported (9-12), respectively. A total and direct bilirubin level was evaluated according to Tietz (13) while indirect bilirubin was calculated by subtraction of direct bilirubin from total bilirubin (12). Evaluation of urea, creatinine, sodium and potassium were performed according to previous reports respectively (14-16).

Statistical analysis

Data obtained from this investigation was statistically analyzed using the one way analysis of variance (ANOVA) (17). Means at the same row have different letters are significantly different.

Results and discussion

Prior to the treatment, the clinical examination revealed that twenty out of thirty cows with a case history of anorexia and loss of condition revealed pyrexia above (41°C), weakness, depression, rumen stasis, increase in respiratory and heart rates, conjunctival and vaginal mucous membranes were ranged from pale in mild cases to dark yellowish or icteric discoloration (Figure 1A) in more progressive cases, in addition to hemoglobinuria (Figure 1B). The microscopic examination of Giemsa stained blood smear divulge double pear shaped (pyriform) of *Babesia* inside RBCs (intra-erythrocytes) in thirteen clinically infected cows (Figure 2). The newly designed primer set succeeded in amplification of 733 bp of 18S rRNA region in all piroplasms. The nucleotide sequence analysis confirmed the presence of *B. bigmina* in the twenty diseased cows. There is no correlation between the number of RBCs containing pyriform *Babesia* and severity of the clinical symptoms, where in seven cases Giemsa stained blood smears give a false negative results that could attributed to the site of blood collection and the thickness of blood film. But, PCR succeeded to discover existence of protozoa even with minute levels of infection as reported by Constable et al. (18).

The sudden onset of pyrexia in this study could be referred by a response to the impact of the toxic substances on thermoregulatory that generated during the metabolism of *Babesia* (18). On the other hand, hemolysis of RBCs leads to anemia and anemic hypoxia resulting in increase in respiratory and heart rates which might be a compensatory mechanism of the body for proper oxygenation of the tissues (18). Hemoglobinuria present in infected animals could be also referred to intense hemolysis related to presence of *Babesia* spp. inside RBCs resulting in hemoglobinemia and therefor hemoglobinuria (19). In current study the observed clinical findings were in accordance with results of previously recorded signs in babesiosis (20,21).



Figure 1: Clinical signs of babesiosis in naturally infected cow showing icteric vaginal mucous membrane (A) and dark red to brown urine (B)



Figure 2: Giemsa-stained blood smear of naturally infected cow showing intra-erythrocytic Pyriform (Pear-shape) of *Babesia bigemina* in pairs (120 X)

Treatment of *Babesia*-infected cows with imidocarb dipropionate in this study led to improvement in the clinical signs in the treated cows with subsiding of high temperature, heart and respiratory rates in addition to the color of urine changed from dark red/brown to slight yellow (normal color) that suggested the success of treatment. Imidocarb dipropionate became the product of choice in treatment and control of babesiosis, in addition to its therapeutic usefulness, it also witnessed to be

effectiveness as preventive when used twice of therapeutic dosage (3). Among its advantages it is the solely babesiacide that continually clears the host of parasites, and cows treated with imidocarb may finish up with a sterile solid immunity (22).

Concerning the hematological findings in this study (Table 1), a significant falloff in the mean of RBCs count, HCT%, Hb concentration and MCHC with an increase in MCV were observed in cows infected with *B. bigemina*

when compared with healthy control animals that indicated a macrocytic hypochromic anemia. These results may be attributed to mechanical impairment caused by binary cleavage of trophozoite intra-erythrocytes (23), production of anti-erythrocyte antibodies directed against structural erythrocytes membrane either infected or the uninfected (24), and due to the increase in erythrophagocytosis by activated macrophages

(25). The same results were obtained previously (6,26,27). The macrocytic hypochromic anemia may be due to increased erythropoiesis and release of a significant number of reticulocytes from the bone marrow which occurred as a response to the hemolytic process (28). MCH values revealed a significant increase in Babesia infected non treated cows when compared with control animals that confirm hemolytic condition (29).

Table 1: Erythrogram and leucogram (means $\pm$ SE) of control non-infected cows, Babesia infected cows before and 7 days after treatment with imidocarb dipropionate 12% by a single I/M injection dose 1.7 mg/kg BW

Parameters	Cows			P-value
	Control	Before treatment	Seven days after treatment	
Hemoglobin (g/dl)	9.91 $\pm$ 0.27 ^a	5.48 $\pm$ 0.23 ^c	7.45 $\pm$ 0.26 ^b	0.01
RBCs ($\times 10^6/\mu\text{l}$)	6.85 $\pm$ 0.21 ^a	3.28 $\pm$ 0.14 ^c	5.00 $\pm$ 0.17 ^b	0.00
Hematocrit (%)	30.03 $\pm$ 0.68 ^a	17.92 $\pm$ 0.74 ^c	22.87 $\pm$ 0.8 ^b	0.00
MCV(fl)	43.84 $\pm$ 0.89 ^b	54.63 $\pm$ 1.68 ^a	45.74 $\pm$ 1.06 ^b	0.01
MCH (pg)	14.47 $\pm$ 0.36 ^b	16.70 $\pm$ 0.4 ^a	14.90 $\pm$ 0.29 ^b	0.03
MCHC (%)	33.00 $\pm$ 0.25 ^a	30.58 $\pm$ 0.44 ^b	32.58 $\pm$ 0.43 ^a	0.01
WBCs ($\times 10^3/\mu\text{l}$)	9.86 $\pm$ 0.32 ^a	5.48 $\pm$ 0.41 ^c	8.5 $\pm$ 0.3 ^b	0.00
Lymphocytes ($\times 10^3/\mu\text{l}$)	5.43 $\pm$ 0.27 ^a	2.35 $\pm$ 0.25 ^c	4.55 $\pm$ 0.23 ^b	0.00
Monocytes ($\times 10^3/\mu\text{l}$)	0.43 $\pm$ 0.02	0.47 $\pm$ 0.02	0.44 $\pm$ 0.02	0.74
Neutrophils ($\times 10^3/\mu\text{l}$)	3.72 $\pm$ 0.09 ^a	2.38 $\pm$ 0.14 ^c	3.22 $\pm$ 0.09 ^b	0.00
Eosinophils ($\times 10^3/\mu\text{l}$)	0.12 $\pm$ 0.006 ^b	0.15 $\pm$ 0.007 ^a	0.14 $\pm$ 0.009 ^{a,b}	0.04
Basophils ($\times 10^3/\mu\text{l}$)	0.14 $\pm$ 0.008	0.12 $\pm$ 0.006	0.12 $\pm$ 0.007	0.83
Platelets ($\times 10^3/\mu\text{l}$)	529 $\pm$ 27.08 ^a	273 $\pm$ 4.82 ^c	422 $\pm$ 21.38 ^b	0.01

**Highly significant difference at $p \leq 0.01$; *significant difference at $p \leq 0.05$, N S: not significant

RBCs: total erythrocytic count, MCV: mean corpuscular volume, MCH: mean corpuscular haemoglobin, MCHC: mean corpuscular hemoglobin concentration, WBCs: white blood cells

Different superscript letters at the same row were significantly different.

Regarding leucogram in this study (Table 1), a significant falloff in WBCs, lymphocyte and neutrophil counts along with a significant increase in eosinophil count was observed in infected cows before treatment compared with control cows. The monocytes and basophils counts were non-significantly changed. One mechanism involved in leucopenia in Babesia infected cows before treatment includes the ability of platelets to bind with activated endothelial cells to interact with leucocytes and induce "secondary capture". The subsequent neutrophil-endothelial interaction could contribute to the decrease in WBCs count (30). Moreover, the mechanisms of neutropenia is yet to be refined whether it's an effect of direct damage to the hematopoietic precursor cells, splenic sequestration, raised neutrophil

adherence, or a series of all (31) as well as neutropenia is common during acute infections (32). Lymphopenia observed in Babesia-infected cows before treatment may be attributed to stress (12). Another reason for neutropenia, lymphopenia and subsequently leukopenia may be attributed to sequestration of leucocytes in the spleen (33). While the significant increase in eosinophils due to infected cows mostly with parasitic tick infestation on skin cause allergic reactions (12). Thrombocytopenia was seen in Babesia infected cows before treatment could be attributed to several mechanisms such as platelet sequestration inside the spleen, destructed platelet as immune mediated disease and progress of disseminated intravascular coagulation (26,27). Following administration

of imidocarb dipropionate to Babesia-infected cows, an improvement in the hematological parameters was recorded which represented by an increase in the mean levels of RBCs count, HCT%, Hb concentration toward the normal values and this may be attributed to the elimination of the *Babesia* spp. from blood of the treated animals (34).

Regarding the biochemical investigations in the present work (Table 2), this disclosed that there was a significant increase in activities of serum ALT and AST of cows infected with *B. bigemina* when compared with control non-infected cows. Also, hepatic cell degeneration resulted from enormous hemolysis may occur in synchronization with hypoxia may lead to increase in serum AST and ALT activities (35).

Erythrocytes also contain AST; therefore, the hemolysis occurred during the disease considered as an important cause of increase serum AST activity (32). Also, there was a significant excess in serum activity ALP of cows infected with *B. bigemina* when compared with healthy non-infected cows, ALP derives from the bile ducts and does not leak from the hepatocyte with increased membrane permeability, so cholestasis probably caused by hepatomegaly could be the reason of the marked increase in ALP activity (32). Also a significant increase of LDH that is most abundant in erythrocyte and hepatic cells can function as a marker for erythrocyte hemolysis and hepatocellular injury (12, 32).

Table 2: Serum liver function parameters (means $\pm$ SE) of control non-infected cows, Babesia infected cows before and 7 days after treatment with imidocarb dipropionate 12% single I/M injection 1.7 mg/ kg BW

Parameters	Cows			P-value
	Control	Before treatment	Seven days after treatment	
ALT (U/L)	31.3 $\pm$ 3.77 ^c	75.2 $\pm$ 3.59 ^a	46.85 $\pm$ 2.55 ^b	0.00
AST (U/L)	65.5 $\pm$ 3.55 ^c	136.5 $\pm$ 5.33 ^a	87.15 $\pm$ 5.18 ^b	0.00
ALP (U/L)	108.3 $\pm$ 5.43 ^c	287.7 $\pm$ 8.34 ^a	184.1 $\pm$ 9.23 ^b	0.00
LDH (U/L)	53.1 $\pm$ 1.02 ^c	69.7 $\pm$ 2.13 ^a	58.91 $\pm$ 0.98 ^b	0.00
T. Proteins (g/dL)	6.78 $\pm$ 0.08 ^a	5.27 $\pm$ 0.09 ^c	5.88 $\pm$ 0.1 ^b	0.00
Albumin (g/dL)	3.54 $\pm$ 0.13 ^a	2.8 $\pm$ 0.08 ^c	3.14 $\pm$ 0.04 ^b	0.01
Globulins (g/dL)	3.24 $\pm$ 0.1 ^a	2.47 $\pm$ 0.1 ^b	2.74 $\pm$ 0.1 ^b	0.00
A/G ratio	1.11 $\pm$ 0.04	1.16 $\pm$ 0.07	1.21 $\pm$ 0.06	0.62
T. Bilirubin (mg/dL)	0.32 $\pm$ 0.02 ^c	1.66 $\pm$ 0.11 ^a	0.72 $\pm$ 0.04 ^b	0.00
D. Bilirubin (mg/dL)	0.18 $\pm$ 0.01 ^b	0.29 $\pm$ 0.02 ^a	0.2 $\pm$ 0.005 ^b	0.00
Ind. Bilirubin (mg/dL)	0.14 $\pm$ 0.01 ^c	1.37 $\pm$ 0.09 ^a	0.52 $\pm$ 0.03 ^b	0.00

**Highly significant difference at $p \leq 0.01$, N S: not significant,

AST: aspartate aminotransferase, ALP: alkaline phosphatase, ALT: alanine aminotransferase, LDH: lactate dehydrogenase and A/G ratio: albumin/ globulins ratio.

Different superscript letters at the same row were significantly different.

Concerning serum proteinogram, a significant decrease in the serum levels of total proteins, albumin and globulins were recorded in cows infected by *B. bigemina* and these could be due to decrease protein production as a result of deprivation of diet protein resulting from anorexia and fever accompanied infection. Also, disturbed hepatic functions and loss of proteins in urine can play a role (36). The present data disclosed a significant rise in levels of serum bilirubin (total, direct and indirect) in cows infected with *B. bigemina* when compared with control cows which may be attributed to

the massive hemolysis occurring throughout the infection period resulting in hypoxia that cause degeneration of the hepatic cell that leads to increase level of serum bilirubin (34, 37).

Regarding kidney function tests, a significant rise in levels of serum urea and creatinine with a significant decrease in both sodium and potassium values in Babesia-infected cows in compare with the control cows. The observed elevation of urea and creatinine levels (Table 3) in Babesia-infected cows could be resulted from kidney dysfunction. This impairment in renal function may be due to necrotizing,

separation of epithelial cells of renal tubules in proximal convoluted tubules and hemoglobin casts (38). The increase in levels of serum creatinine and urea could be also referred to systemic hypotension that occurs in *Babesia* infection lead to kidney vasoconstriction that have be the most principal cause of kidney hypoxia and more inculcate than hemog-

lobinuria in damaging of renal tissue, also hemolytic anemia may participate to deficient oxygenation lead to degenerative changes in the cytoplasm of the renal tubules lining epithelial cells in association with perivascular mononuclear leucocytic cellular infiltrations mainly lymphocytes and macrophages (35).

Table 3: Serum kidney function parameters (means $\pm$ SE) of control non-infected cows, *Babesia* infected cows before and 7 days after treatment with imidocarb dipropionate 12% single I/M injection 1.7 mg/ kg BW

Parameters	Cows			P-value
	Control	Before treatment	Seven days after treatment	
Urea (mg/dL)	17.7 $\pm$ 1 ^b	42.1 $\pm$ 2.37 ^a	18.5 $\pm$ 0.35 ^b	0.00
Creatinine (mg/dL)	1.1 $\pm$ 0.03 ^b	1.98 $\pm$ 0.13 ^a	1.3 $\pm$ 0.06 ^b	0.01
Sodium (mmol/L)	141.5 $\pm$ 0.7 ^a	137.05 $\pm$ 0.76 ^b	140.7 $\pm$ 0.48 ^a	0.00
Potassium (mmol/L)	4.2 $\pm$ 0.04 ^a	3.9 $\pm$ 0.03 ^c	4.09 $\pm$ 0.03 ^b	0.00

**Highly significant difference at $p \leq 0.01$

Different superscript letters at the same row were significantly different.

The hyponatremia observed in this study in cows infected with *Babesia* may be produced by reduction of renal perfusion and hypotension, that have been percept throughout the course of babesiosis as described by Matijatko et al. (39). In this status, reduction of glomerular filtration invigorates the renin angiotensin aldosterone system, lead to reservation of sodium and water. Furthermore, activation of antidiuretic hormone liberate in hypotension cases causes reduced water excretion. The dilution of sodium despite of the retention leads to hyponatremia (40).

Hypokalemia noticed in this study in cows infected with *B. bigemina* when compared with healthy non-infected cows could be due to forfeiture of potassium ions by the alimentary tract or kidneys, or from potassium translocation into intracellular fluids (41). Also degenerative and necrotic alterations in the renal proximal tubules of may contribute in the development of hypokalemia (42).

After administration of imidocarb dipropionate to *Babesia*-infected cows, there was an improvement in biochemical parameters that indicates recovery from the possible liver and kidney damage. The present results were in agreement with Sevinc et al. (43), while they were in disagreement with results of Kumar et

al. (44). The disagreement may be due to different animal species and different blood parasite subspecies.

Conclusion

It could be concluded that molecular detection of *B. bigemina* is more sensitive than blood smear. The hematological and biochemical parameters are adversely affected in *B. bigemina* naturally infected cows, which could be improved by single dose of imidocarb.

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Conflict of interest

The authors declare that they have no competing interests.

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