EFFECTS OF IMIDOCARB DIPROPIONATE AND ALPHA-LIPOIC ACID IN DOGS EXPERIMENTALLY INOCULATED WITH *BABESIA CANIS VOGELI*

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MAY, 2018

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DEPARTMENT OF VETERINARY MEDICINE,
AHMADU BELLO UNIVERSITY,
ZARIA, NIGERIA

MAY, 2018

DECLARATION

I declare that the work in this Thesis entitled **"EFFECTS OF IMIDOCARB DIPROPIONATE AND ALPHA-LIPOIC ACID IN DOGS EXPERIMENTALLY INOCULATED WITH *BABESIA CANIS VOGELI*"** has been performed by me in the Department of Veterinary Medicine. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any institution.

Ajoke Modupeoluwa EHIMIYEIN _____ _____

Signature **Date**

CERTIFICATION

This Thesis entitled **"EFFECTS OF IMIDOCARB DIPROPIONATE AND ALPHA-LIPOIC ACID IN DOGS EXPERIMENTALLY INOCULATED WITH *BABESIA CANIS VOGELI*"** by AJOKE MODUPEOLUWA EHIMIYEIN meets the regulations governing the award of the degree of Doctor of Philosophy in Small Animal Infectious Diseases of Ahmadu Bello University, and is approved for its' contribution to knowledge and literary presentation.

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DEDICATION

I dedicate this Thesis to the Glory of God Almighty and to my parents, Mr. Meshack Oluwade Odeja and late Mrs. Oluwafunmilayo Olaore Odeja, who believed so much in me.

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ABSTRACT

The aim of the study was to detect, identify and characterise the *Babesia canis* spp. that was inoculated, and evaluate the effects of imidocarb dipropionate and alpha-lipoic acid (ALA) on the clinical manifestations, haemato-biochemical and oxidative stress changes in Nigerian indigenous dogs experimentally inoculated with *Babesia canis vogeli*. The parasite inoculated was identified parasitologically and molecularly characterised from blood sample of a *Babesia canis* spp-positive donor using polymerase chain reaction before experimental inoculation into the experimental dogs. Twenty-one male Nigerian indigenous dogs, aged 8 - 12 months and weighing 8 - 12 kg, were divided into 5 groups: group I (uninfected untreated, n = 4), group II (infected untreated, n = 6), group III (infected + imidocarb, n = 3), group IV (infected + ALA, n = 5) and group V (infected + imidocarb + ALA, n = 3), respectively. Infected + imidocarb dogs were administered subcutaneously with imidocarb dipropionate at 6 mg/kg and repeated 14 days after; infected + ALA dogs were given alpha-lipoic acid (15 mg/kg, orally) daily, throughout the experimental duration; and infected + imidocarb + ALA dogs were administered with a combination of imidocarb + ALA at the same doses and routes. Clinical signs and vital parameters were recorded daily. Blood samples were collected: in tripotassium ethylenediaminetetraacetic acid (EDTA) and used for haematology, erythrocyte osmotic fragility and erythrocyte malondialdehyde concentration; plain bottles were used to obtain serum for the biochemical analyses and acid citrate dextrose for platelet glutathione peroxidase. The samples were obtained before inoculation and at days 11 post-inoculation (PI), post-treatment (PT) days 8, 15 and 43, respectively. Treatment commenced at day 12 PI. Biochemical parameters activities of liver function enzymes and concentrations of oxidative stress biomarkers were analysed using commercial kits. The result showed that 576 bp gene sequence gel electrophorogram for *B. canis vogeli,* and mild clinical manifestations were observed.

Decreased packed cell volume below the normal range was recorded in all the infected groups at days 11 PI and 15 PT, except in the infected + imidocarb + ALA group at day 15 PT (41.13 ± 2.96%). Infected + imidocarb + ALA dogs had the highest erythrocyte count at PT days 15 (6.10 ± 0.44 x 10^{12}/L) and 43 (6.99 ± 0.56 x 10^{12}/L). The haemoglobin concentration in all the infected groups decreased at days 11 PI and 15 PT, except in the infected + imidocarb + ALA group at day 15 PT (13.80 ± 0.91 g/dL). Lower ($P < 0.05$) mean corpuscular haemoglobin concentrations were recorded in the infected + imidocarb at day 11 PI (32.50 ± 0.60 g/dL), and in all the infected groups at days 8 PT and 43 PT. Decreased neutrophil and increased lymphocyte counts were obtained throughout the study period. Marked thrombocytopenia was recorded in all the infected groups at day 11 PI. At PT days 8, 15 and 43, the infected untreated dogs had the highest percentage (81.14 ± 4.34%, 86.31 ± 1.39%, 68.76 ± 5.25%) haemolysis at 0.5% NaCl concentration while the infected + imidocarb + ALA dogs had the lowest (56.43 ± 0.80%, 34.93 ± 0.37%, 56.39 ± 2.36%). The bilirubin concentration in the infected untreated dogs (14.45 ± 3.62 µmol/L) dogs was higher ($P < 0.05$) than the value recorded in the uninfected untreated dogs (2.69 ± 1.02 µmol/L) at day 15 PT. Lowest albumin concentrations were recorded in the infected untreated dogs (2.20 ± 0.47 g/dL) at day 11 PI, infected + ALA (1.61 ± 0.29 g/dL) at day 8 PT, infected + imidocarb (2.22 ± 0.40 g/dL) at day 15 PT, and infected untreated (2.05 ± 0.15 g/dL) at day 43 PT. Infected + imidocarb + ALA had normal albumin concentration; except at day 11 PI (2.23 ± 0.49 g/dL). Erythrocyte malondialdehyde concentration was significantly ($P < 0.05$) higher in all the infected groups at days 11 PI, 8 PT, 15 PT and 43 PT, respectively. Lowest serum superoxide dismutase activities were recorded in the infected untreated at PT days 15 (0.33 ± 0.05 UL) and 43 (1.83 ± 0.11 UL). A significant ($P < 0.05$) increase in concentration of tumour necrosis factor-alpha was obtained in all the infected groups at days 11 PI, 8 PT, 15 PT and 43 PT. The study showed that *Babesia canis*

vogeli caused mild clinical signs. It induced macrocytic hypochromic anaemia at day 8 PT, MCV (76.50 ± 1.12 fl, 77.00 ± 0.74 fl, 72.00 ± 0.85 fl, 74.73 ± 1.63 fl) and MCHC (30.18 ± 0.28 g/dL, 29.87 ± 0.38 g/dL, 31.16 ± 0.47 g/dL, 30.40 ± 0.25 g/dL), neutropenia, lymphocytosis, marked thrombocytopenia and increased haemolysis. The study showed that treatment of experimentally-induced canine babesiosis with imidocarb dipropionate and/or ALA ameliorated the clinical signs, haemato-biochemical and oxidative stress changes in Nigerian indigenous dogs. The amelioration was more pronounced when treated with combination of imidocarb and ALA, resulting in a synergistic effect that reduced the severity of the clinical signs, haemato-biochemical and oxidative stress changes associated with canine babesiosis. A combination of imidocarb and ALA may be a promising treatment for canine babesiosis.

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LIST OF ABBREVIATIONS

A -	Absorbance
ACD -	Acid citrate dextrose
ADP -	Adenosine diphosphate
ATP -	Adenosine triphosphate
ALT -	Alanine aminotransferase
A/G -	Albumin/globulin ratio
ALP -	Alkaline phosphatase
ANOVA -	One-way analysis of variance
α-KG -	Alpha-ketoglutarate
ALA -	Alpha-lipoic acid
AST -	Aspartate transaminase
A-HRP -	Avidin-horseradish peroxidase
B. bigemina -	*Babesia bigemina*
B. bovis -	*Babesia bovis*
B. canis -	*Babesia canis*
B. canis canis -	*Babesia canis canis*
*Bc*28 gene -	*Babesia canis* 28 gene
B. canis rossi -	*Babesia canis rossi*
*Br*EMA 1 -	*Babesia rossi* erythrocyte membrane antigen 1
B. canis vogeli -	*Babesia canis vogeli*
B.conradae -	*Babesia conradae*
B. divergens -	*Babesia divergens*
B. gibsoni -	*Babesia gibsoni*
BLAST -	Basic Local Alignment Search Tool

B. microti -	*Babesia microti*
BCG -	Bromocresol green
BUN -	Blood urea nitrogen
CARS -	Compensatory anti-inflammatory response syndrome
Cmax -	Maximum plasma concentrations
CRP -	C-reactive protein
CST3 -	Cystatin C
C/EBPβ -	CCAAT/enhancer binding protein
$CaCl_2.6H_2O$ -	Calcium chloride hexahydrate
$C_8H_{14}O_2S_2$ -	1, 2-dithiolane-3-pentanoic acid
CGS -	Citrate glucose saline
Ca^{2+} -	Calcium ion
CK -	Creatine kinase
CO_2 -	Carbon dioxide
Cu^{2+} -	Copper ion
D -	Day of onset of clinical manifestations
DNA -	Deoxyribonucleic acid
DHLA -	Dihydrophilic acid
ELISA -	Enzyme-linked immunosorbent assays
EDTA -	Ethylenediaminetetraacetic acid
ETS -	EDTA/Tris/Saline
FASTA -	Fast adaptive shrinkage thresholding algorithm
Fe^{3+} -	Ferric cation
Fe^{2+} -	Ferrous ion

FTA -	Flinders technology associates
γ-GT -	Gamma-glutamyl transferase
G6PDH -	Glucose-6-phosphate dehydrogenase
GLDH -	Glutamate dehydrogenase
GPT -	Glutamate pyruvate transaminase
GSH -	Glutathione
GPx -	Glutathione peroxidase
GSSH -	Oxidised glutathione
H^+ -	Hydrogen ion
Hb -	Haemoglobin
HCL -	Hydrogen chloride
Hg^{2+} -	Mercury ion
HK -	Hexokinase
HNO_3 -	Nitric acid
H_2O -	Water
H_2O_2 -	Hydrogen peroxide
IFAT -	Indirect fluorescent antibody test
IFCC -	International Federation of Clinical Chemistry
Ig -	Immunoglobulins
IL-1 -	Interleukin-1
IL-6 -	Interleukin-1
IL-4 -	Interleukin-4
IL-10 -	Interleukin-10
IL-6 -	Interleukin-6
IMHA -	Immune-mediated haemolytic anaemia

I. persulcatus -	*Ixodes persulcatus*
ITS -	Internal transcribed spacer
K^+ -	Potassium ion
KCl -	Potassium chloride
LAMP -	Loop-mediated isothermal amplification
LD_{50} -	Lethal dose
MCH -	Mean corpuscular haemoglobin
MCHC -	Mean corpuscular haemoglobin concentration
MCV -	Mean corpuscular volume
MDA -	Malondialdehyde
MEGA -	Molecular evolutionary genetics analysis
Mg -	Magnesium
Mg^{2+} -	Magnesium ion
Mg-ATP -	Magnesium adenosine triphosphate
$MgCl_2.6H_2O$ -	Magnesium dichloride hexahydrate
MODS -	Multiple organ dysfunction syndrome
MSA -	Merozoite surface antigens
NaCl -	Sodium chloride
NAD -	Nicotinamide adenine dinucleotide
NADH -	Nicotinamide adenine dinucleotide
$NADP^+$ -	Nicotinamide adenine dinucleotide phosphate
NADPH -	Nicotinamide adenine dinucleotide phosphate
$NaHCO_3$ -	Sodium hydrogen carbonate
$NaNO_2$ -	Sodium nitrite
NaOH -	Sodium hydroxide

NF-Kβ -	Nuclear factor- kappa β
NH_3 -	Ammonia
NH_4 -	Ammonium cation
Nitro-PAP -	Nitro-p-Aminophenol
NJ -	Neighbour-joining
NKC -	Natural killer cells
NLR -	Neutrophil lymphocyte ratio
OH^- -	Hydroxyl radical
O_2 -	Oxygen
O_2^- -	Superoxide anion radical
P -	Phosphorus
Pb^{2+} -	Lead ion
PCR -	Polymerase chain reaction
PCV -	Packed cell volume
PI -	Post-inoculation
PPP -	Plasma poor platelet
PR -	Pulse rate
PRP -	Platelet rich plasma
PT -	Post-treatment
qPCR -	Quantitative PCR
R-ALA -	R- lipoic acid
R. annulatus -	*Rhipicephalus annulatus*
RBC -	Red blood cells
rBg -	Recombinant antigen of *Babesia gibsoni*
R. bursa -	*Rhipicephalus bursa*

R. decolaratus -	*Rhipicephalus decolaratus*
R. evertsi -	*Rhipicephalus evertsi*
RFLP -	Restriction fragment length polymorphism
R. geiyi -	*Rhipicephalus geiyi*
RLB -	Reverse-line blot
RNA-	Ribonucleic acid
RNS -	Reactive nitrogen species
ROH -	Alcohol
RT -	Rectal temperature
ROOH -	Hydroperoxide
ROS -	Reactive oxygen species
RR -	Respiratory rate
RT -	Rectal temperature
S-ALA -	S-lipoic acid
SEM -	Standard error of mean
SOD -	Superoxide dismutase
SIRS -	Systemic inflammatory response syndrome
SPA -	Soluble parasite antigens
TAE -	Tris acetate-EDTA
TNF-α -	Tumour necrosis factor-alpha
TP -	Total protein
TPB -	Turbidimetric tetraphenylborate
TRAP -	Thrombospondin-related adhesive protein
uCRP -	Urinary C-reactive protein
uIgG -	Urinary immununoglobulin G

uRBP -	Urinary retinol binding protein
UV -	Ultraviolent
VEGF -	Vascular endothelial growth factor
WBC -	White blood cells
WHO -	World Health Organisation
XO -	Xanthine oxidase
Zn^{+} -	Zinc ion

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Babesia species are tick-borne Apicomplexan erythrocytic parasites (Slodki *et al.,* 2011; Varloud *et al.,* 2018) that infect variety of domestic animals, including dogs (Paulauskas *et al.,* 2014), wild animals (Dawood *et al.,* 2013) and humans (Zhou *et al.,* 2014; Mørch *et al.,* 2015). *Babesia* sp. was first identified in 1988 by Viktor Babes (Solano-Gallego *et al.,* 2016) in red blood cells (RBC) of cattle in Romania as the cause of bovine haemoglobinuria or red water fever. Babesiosis is fast becoming a disease of public health importance (Zobba *et al.,* 2011) and it causes major economic losses (Hasle *et al.,* 2010), affecting adversely the health of many domestic animals, including cattle and sheep (Tavassoli *et al.,* 2013), pigs (Zobba *et al.,* 2011) and dogs (Schoeman, 2009). It is a tick-borne disease transmitted by *Ixodidae* ticks (Slodki *et al.,* 2011), including *Rhipicephalus sanguineus* (dog), *R. decolaratus* (cattle), *R. geiyi* (cattle), *R. annulatus* (cattle), *R. evertsi* (cattle, horse, zebra) *R. bursa* (sheep and goat) *Ixodes ricinus* (cattle, sheep, deer) and *I. persulcatus* (human, sheep, dog) (Halos *et al.,* 2013; Tavassoli *et al.,* 2013). The organism is an intra-erythrocytic parasite of the genus, *Babesia*. Different species of *Babesia spp.* (Bajer *et al.,* 2014; Laha *et al.,* 2015; Hamšíková*et al.,* 2016), such as *B. divergens, B. bovis* and *B. bigemina* affect animals, including cattle (Mosqueda *et al.,* 2012) and deers (Zanet *et al.,* 2014; Anderson *et al.,* 2016); *B. microti* affects smaller mammals (Hersh *et al.,* 2014); *B. microti* and *B. divergens* are causative agents in humans (Yabsley and Shock, 2012; Zhou *et al.,* 2013; Wei *et al.,* 2016); and *B. canis* in dogs (Fourie *et al.,* 2013; Salem and Farag, 2014; Koster *et al.,* 2015; Navarro *et al.,* 2015; Solano-Gallego *et al.,* 2016). Canine babesiosis, also known as 'malignant jaundice or bilious fever' (Penzhorn, 2011; Penzhorn *et al.,* 2017), has been documented worldwide (Schaarschmidt *et al.,* 2006). It is an

emerging infectious disease in Europe (Solango-Gallego and Baneth, 2011) that is caused by two distinct species, based on the morphology characterization of the parasite in the RBC (Solano-Gallego and Baneth, 2011), including the large (2.5 - 5.0 µm) *Babesia canis*; and the small (1.0 - 2.5 µm), *B. gibsoni* and *B.conradae* (Singh *et al.,* 2014; Laha *et al.,* 2015; Zygner *et al.,* 2015). *Babesia canis* is further divided into sub-species (Duarte *et al.,* 2011; Torti *et al.,* 2014); *B. canis canis,* transmitted by the tick *Dermacentor reticulatus* in Europe; *B. canis vogeli* (Barker *et al.,* 2012; Halos *et al.,* 2013), transmitted by *Rhipicephalus sanguineus* in tropical and sub-tropical regions; and *B. canis rossi*, transmitted by *Haemophysalis leachi* in South Africa (Bashir *et al.,* 2009). The three sub-species were previously considered to be morphologically identical (Solano-Gallego *et al.,* 2016), but differ in the severity of clinical manifestations which they induce, their tick vectors, genetic characteristics and geographic distributions. They are, therefore, currently considered separate species (Solano-Gallego and Baneth, 2011). *Babesia gibsoni* occurs in Asia, North America, Northern and Eastern Africa, Australia and Europe (Salem and Farag, 2014; Wei *et al.,* 2014).

Concurrent infections of *B. canis rossi* occur with *B. canis vogeli* (Sasaki *et al.,* 2007; Kostro *et al.,* 2015; Nalubamba *et al.,* 2015), and also *B. canis canis* with *B. canis rossi* in an untraveled 6-month-old femal Alsatia dog presented to the Veterinary Clinic of the National Veterinary Research Institute, Vom, Plateau State, Nigeria (Sasaki *et al.,* 2007; Kamani *et al.,* 2010). Several authors have reported the endemicity of *Babesia canis* in Nigeria including Oduye and Otesile (1977), who reported that haemoparasites accounts for 52% of anaemia in dogs in Ibadan. The prevalence of *Babesia canis* varies from one part of Nigeria to another (Egege *et al.,* 2008). Oduye and Dipeolu (1978) reported that *Babesia canis* accounted for 53% parasitaemia in 500 dogs in Ibadan, Sarror *et al.* (1979) recorded

22% prvelence of *Babesia canis*-infected dogs in 254 dogs in Zaria and Odewunmi and Uzoukwu (1979), 55.17% prevalence in 116 dogs in Enugu. Lawal *et al.* (2001) reported 40.3% prevalence of *Babesia canis* in exotic breeds of dogs in Zaria. Egege *et al.* (2008) reported highest prevalence rate of 73.3% of canine babesiosis between May and July with Nigeria indigenous dogs more at risk than exotic dogs. The breed specific mortality rate for babesiosis was higher in Nigerian indigenous dogs (54.3%) than in exotic dogs (53.4%) (Egege *et al.,* 2008). Takeet *et al.* (2017) detected *Babesia rossi* in dogs presented for routine examination in Veterinary Hospitals in Abeokuta, Nigeria. Canine babesiosis has been reported to cause 9.5% mortality in dogs in and around Effurun/Warri Municipality of Delta State, Nigeria (Shima *et al.,* 2014).

The clinical manifestations observed in canine babesiosis vary, depending on the sub-species, the immune status of the host, the age and geographical location. *Babesia canis vogeli* causes sub-clinical, mild, or moderate form (Defauw *et al.*, 2012; Gabrielli *et al.*, 2015), while *B. canis canis* infection is more severe and typically present with acute form of babesiosis (Tiskina *et al.,* 2016), which is characterised by fever and haemolytic syndromes, general weakness, pale mucous membrane and depression. *B. gibsoni* causes more severe clinical manifestations than *B. canis,* which may affect multiple organs (Aysul *et al.,* 2013). The common clinical manifestations and haematological findings associated with canine babesiosis include fever, inappetence, haemoglobinuria, bilirubinuria, haemolytic anaemia, thrombocytopenia, splenomegaly, hepatomegaly, jaundice, lymphadenopathy and eventually death (Abdullahi *et al.,* 1990; Schoeman, 2009; Salem and Farag, 2014). Pale mucous membrane, icterus and diarrhoea were reported in dogs with babesiosis (Egege *et al.,* 2008).

The different drugs conventionally used for the treatment of canine babesiosis include diminazene aceturate, imidocarb dipropionate and tryphan blue. The drugs have different side-effects and precaution must be taken before their administration. Diminazene aceturate has been used in the treatment of babesiosis but it is unable to eliminate *B. gibsoni*, thus causing relapse (Lin and Huang, 2010; Koster *et al.,* 2015; Kumara, 2016). It is highly toxic, causing signs associated with hepatoxicity, nephrotoxicity and haemorrhage in the cerebellum, and necrosis at the site of injection (Iguchi *et al.,* 2014; Baldissera *et al.,* 2015; Baldissera *et al.,* 2016). Imidocarb dipropionate is the drug of choice for treating large *Babesia* spp. (Mosqueda *et al.,* 2012; Torbica *et al.,* 2013; Nalubamba *et al.,* 2015; Rocio *et al.,* 2017), this is because it has not only the ability to eliminate the parasites within 2 - 3 days after administration (Torbica *et al.,* 2013), it has been used in the treatment and prophylaxis of babesiosis (Mosqueda *et al.,* 2012); although the mode of action is not clear. It has been proposed to interfere with the production or use of polyamine (Nwoha and Omamegbe, 2015), or the prevention of entry of inositol into the parasitised RBC, starving the parasites (Wise *et al.,* 2013). There is usually clinical relapse when imidocarb dipropionate is used in the treatment of small *Babesia* spp. A combination of an anti-protozoan drug (atovaquone) and an antibiotic (azithromycin) has been effective in the treatment of *B. gibsoni* and human babesiosis (Usatii *et al.,* 2014). However of recent, drug resistant mutants of *B. gibsoni* have developed against the protocol (Lin *et al.,* 2012).

Parasitic infection is detrimental to the host, affecting the immune system (Van de Crommenacker *et al.,* 2011). Increased generation of excessive reactive oxidants is released by the host to combat infection. Reactive oxygen species including O_2^-, OH^-, O_2, and H_2O_2 occur under normal physiological conditions in the body; and they are toxic. If ROS level supersedes that of the system's antioxidants, it results into oxidative stress. Oxidative stress

is an imbalance between ROS-generating and scavenging activities. It occurs due to inadequate antioxidants or excessive production of ROS, inducing tissue damage (Radwan *et al.*, 2013). Oxidative stress plays an important role in the pathogenesis of diseases (Mahjoub and Masrour-Roudsari, 2012) and impairment of organ functions; and, thus, plays critical role in the overall survival of the animals (Crnogaj *et al.*, 2010).

In babesiosis, there is an increased generation of ROS, resulting in increased malondialdehyde (MDA) concentration in the blood of infected animals (Salem *et al.*, 2016; Crnogaj *et al.*, 2017). Thus, oxidative stress occurring in babesiosis enhances lipid peroxidation in body cells, including the RBCs, with the attendant increased haemolysis (Osman and Gaadee, 2013). In order to alleviate the excessive ROS production, antioxidants are administered to scavenge ROS and, thus, reduce tissue destruction mediated via ROS-induced lipid peroxidation (Rao *et al.*, 2011; Kurutas, 2016). Pathogenesis of anaemia in babesiosis is partially derived from oxidation of the RBC (Radwan *et al.*, 2013). In a study conducted by Yukio *et al.* (2002), vitamins E and C (Afolabi *et al.*, 2012) have been used to alleviate the oxidative stress by increasing the antioxidants in RBCs in babesiosis.

Toxic ROS are released as an attempt by the host to combat the infection. Antioxidants are non-enzymatic and enzymatic antioxidants. The enzymatic antioxidants are superoxide, catalase, and glutathione peroxidase, involved in scavenging the ROS (Satish and Dilipkumar, 2015). Dietary antioxidants are non-enzymatic antioxidants and are essential nutrients that inhibit the oxidation of important biological tissues and suppress ROS toxicity. When the total amount of antioxidants is insufficient to scavenge the ROS, oxidative damage occurs. This damage contributes to the onset and progression of chronic

diseases, such as neuro-degenerative diseases, cardiovascular diseases and cancer (Yu and Schellhorn, 2013). Administration of antioxidants has different advantages, including speedy recovery from diseases, increase in the overall antioxidant status, decrease in the severity of the disease, prevention of a relapse; and, consequently, reduction in the cost of treatment (Yu and Schellhorn, 2013). The different types of non-enzymatic antioxidants include zinc, selenium, vitamins C and E, and alpha-lipoic acid (ALA) (Abbas *et al.,* 2014).

Alpha-lipoic acid (ALA) was first isolated in 1951 by Reed *et al.* (1951) as a catalytic agent, associated with pyruvate dehydrogenase. It is unique in its ability to act as an antioxidant in fat- and water-soluble tissues in both its oxidised and reduced forms (Uchendu *et al.,* 2014). It is also readily absorbed from an oral dose and has the ability to cross the blood-brain barrier with ease (Shilpi *et al.,* 2015). Due to these advantages and its low toxicity, ALA is potentially an effective therapeutic and anti-inflammatory agent in clinical conditions, associated with ROS-induced tissue damages (Andrea *et al.,* 2015). Antioxidant properties of ALA were discovered by Rosenburg and Culik (1959), who reported that it prevented both scurvy symptoms in vitamin C-deficient guinea pigs, and vitamin E deficiency in rats fed a diet lacking vitamin E.

Podda *et al.* (1994) reported that ALA also prevents symptoms of vitamin E deficiency in mice fed a vitamin E-deficient diet; however, with no effect on sustaining vitamin E tissue concentrations (Podda *et al.,* 1994). ALA is a universal antioxidant (Packer *et al.,* 1995) because of its role in the following processes: specificity of ROS scavenging, metal-chelating activity, interaction with other antioxidants, gene expression and ameliorating properties in neurotoxicity (Paetau-Robinson *et al.,* 2013; Shilpi *et al.,* 2015). The lethal dose (LD_{50}) of ALA was determined as 400 - 500 mg/kg after an oral dosage in dogs

(Packer *et al.*, 1995). Several studies have shown that ALA may suppress pro-inflammatory effects of cytokines, including tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interferon gamma (IFN-$_\gamma$) (Yan *et al.*, 2014). ALA has also been shown to reduce body pro-inflammatory responses, including immunoglobulins (Igs), which are antibody proteins that play a major role in antibody-mediated immunity of the host defence mechanism (Paim *et al.*, 2013).

1.2 Statement of Research Problem

Haemoparasitic diseases of animals are the most important causes of major economic losses, especially in developing countries (Osman and Gaadee, 2013; Koster *et al.*, 2015). Canine babesiosis is one such disease that impairs the body functions of animals and eventually causes death (Silva, 2016). This study was designed to shed more light into the use of ALA as an antioxidant in combination with other conventional therapies for babesiosis in dogs. The cost of prophylaxis and therapeutic treatment of canine babesiosis is high as most of the drugs used are expensive, often associated with many side-effects. Although several drugs have been used to treat babesiosis, no single drug is sufficient for the treatment of babesiosis, especially *B. gibsoni*. The relapses frequently occur (Lin *et al.*, 2012), therefore, in order to increase its efficacy, there was the need to explore the possibility of using agents that may potentiate or act synergistically with the conventional chemotherapeutic agents.

1.3 Justification of the Study

Babesiosis is a zoonotic and emerging disease caused by an intra-erythrocytic protozoan of the *Babesia* spp. (Zhou *et al.*, 2014). It is usually transmitted by ticks depending on the strain and spp. Canine babesiosis can be caused by different species, including large

Babesia spp. (*Babesia canis canis, B. canis vogeli and B. canis rossi*) and small *Babesia* spp. (*B. gibsoni* and *B. microti*) (Bohm *et al.,* 2006). The clinical manifestations in dogs depend on the strain of the *Babesia spp.*, the type of vector, host's immune system and age of the animal, and it present in different forms including hyperacute, acute and chronic or sub-clinical babesiosis. The severity also depends on the form of babesiosis manifested. Diagnosis is usually based on the history, clinical manifestations and blood smear examination. Microscopic examination of *Babesia spp.* is limited by its low sensitivity and cannot differentiate the different species of the parasite. It can be used to differentiate between large and small *Babesia* spp. Although it has high specificity to differentiate *Babesia canis, Babesia canis vogeli* is less commonly detectable microscopically (Solano-Gallego and Banett, 2011; Solano-Gallego *et al.,* 2016). It is also difficult to view the parasite in blood smear in *Babesia* spp. that has low parasitaemia or from animals, exhibiting the chronic or sub-clinical form (Ybanez *et al.,* 2018). Thus, the use of more sensitive method of diagnosis is necessary in order to differentiate to species level. Visualisation of large *Babesia* spp. has been documented by Bohm *et al.* (2006), who reported that capillary parasitaemia is higher than venous parasitaemia because *B. canis rossi* adhere to capillary endothelium. Sequestration of the parasite results in an interaction between the parasite-derived antigen on the parasitised RBC surfaces and endothelial receptors (Bohm *et al.,* 2006).

Relapses in the treatment of canine babesiosis have been reported by Koster *et al.* (2015). Several drugs and drug combinations have been used in the treatment of canine babesiosis with little achievements (Solano-Gallego *et al.,* 2016). Imidocarb dipropionate injections at 6 mg/kg subcutaneously or intramuscularly two weeks apart is the drug of choice for canine babesiosis caused by large *Babesia* spp., but not effective against small *Babesia* spp. (*B.*

gibsoni and *B.microti*-like spp.). This drug has exhibited several side-effects, which include anorexia, hypersalivation, epiphora, abdominal pain, vomiting and diarrhoea. Overdose of imidocarb dipropionate has been associated with nephrotoxicity (Solano-Gallego *et al.*, 2016), and it distrupts the synthesis of nucleic acids (Sakar and Sakar, 1999). Torbica *et al.* (2013) reported a rapid improvement in dogs with uncomplicated babesiosis (Torbica *et al.*, 2013). Fever disappears 24 h post-treatment, while haemoglobinuria disappears within 48 h. *Babesia spp.* is eliminated from RBC within 2 - 3 days after initial treatment (Torbica *et al.*, 2013). It is also used as a prophylaxis, protecting reoccurrence of babesiosis in dogs for 30 days (Vercammen *et al.*, 1996).

Diminazene aceturate administered singly at 3.5 mg/kg intramuscularly is effective against *B. canis*, but not in the treatment of *B. gibsoni*. Although it reduces parasitaemia, morbidity and mortality, neurological signs are some of the effects of diminazene aceturate overdose (Solano-Gallego *et al.*, 2016). Combination of atovaquone (13.5 mg/kg every 8 h with fatty food) and azithromycin (10 mg/kg orally for 10 days) is effective against *B.* spp., including *B. conradae* and *B.microti-like* spp. (Di Cicco and Birkenheuer, 2012). Clindamycin, diminazene and imidocarb dipropionate combination has been reported to be more effective, compared to a combination of atovaquone and azithromycin (Lin *et al.*, 2012). Doxycycline at 10 mg/kg/day orally reduces the severity of the clinical manifestations, thus reducing morbidity, mortality usually associated with *B. canis* and *B.gibsoni* infections (Lin and Huang, 2010). Clindamycin at 25 mg/kg every 12 h for 14 days administered orally has been used in the treatment of *B. gibsoni* infection, and it reduces the severity of the clinical manifestations and laboratory abnormalities (Wulansari *et al.*, 2003).

Several other combinations have been used in the treatment of *B. gibsoni* infection, which include combination of clindamycin (11 mg/kg every 12 h, orally), metronidazole (15 mg/kg every 12 h, orally) and doxycycline (5 mg/kg every 12 h, orally); or enrofloxacin (2.5 mg/kg every 12 h, orally), metronidazole (5 - 15 mg/kg every 12 h, orally) and doxycycline (7 - 10 mg/kg every 12 h, orally) (Suzuki *et al.,* 2007; Lin and Huang, 2010). Combination of atovaquone and proguanil (Malarone®), an antimalaria drug, singly or in combination with doxycycline has been effective in the treatment of *B. gibsoni* infection (Iguchi *et al.,* 2014). Other drugs used in the treatment of babesiosis include trypan blue solution, pentamidine and quinuronium sulphate; experimental treatment with artesunate, plant extracts or tick peptides have been used (Jacobson *et al.,* 1996; Subeki *et al.,* 2004; Tsuji *et al.,* 2007; Ayoob *et al.,* 2010; Goo *et al.,* 2010; Solano-Gallego *et al.,* 2016). Buparvaquone at 5 mg/kg administered after 48 h has proven to be more effective than atovazone in dogs naturally infected with *B. microti*-like spp. in Spain (Checa *et al.,* 2015).

Oxidative stress plays a major role in the pathogenesis of canine babesiosis (Crnogaj *et al.,* 2010; Esmaeilnejad *et al.,* 2014; Kucukkurt *et al.,* 2014). It is usually associated more with the host's inflammatory responses to the parasites than the parasite itself (Matijatko *et al.,* 2007; Schetters *et al.,* 2009). Oxidative stress causes an imbalance in the host pro-inflammatory systemic response and a compensatory modulatory response, accompanied by oxidative injury to tissues (Zygner *et al.,* 2014). These releases ROS and reactive nitrogen species (RNS) which are potent oxidants, causing lipid peroxidation and nitration, leading to generation of free radicals that further damage proteins, membranes and nucleic acids (Crnogaj *et al.,* 2017). Therefore, supplementation with antioxidants scavenges the ROS and prevents tissue damage. Hamid *et al.* (2014) reported the use of combination of

imidocarb dipropionate and vitamin E in amelioration of the clinical signs associated with babesiosis.

Alpha-lipoic acid is an amphiphilic antioxidant that acts in the membrane as well as in the cytosol (Gomes and Negrato, 2014). It has the ability to cross blood-brain barrier, maintaining the integrity of many cells during exposure to oxidative stress (Shay *et al.*, 2009). It has protective ability both in the reduced and oxidised forms. ALA has been beneficial in a myriad of diseases: improvement of endothelial dysfunction (Wray *et al.*, 2012) to reduce oxidative stress post-exercise (McNeilly *et al.*, 2011), prevention and inhibition of atherosclerosis (Gomes and Negrato, 2014), Down syndrome (Lott *et al.*, 2011), Alzheimer's disease (Moreira *et al.*, 2007), hypertension (Vasdev *et al.*, 2000), cognitive dysfunction and some types of cancer (Al-Abdan, 2012). It has also been reported in the treatment of diabetic neuropathy, cardiac autonomic dysfunction and nerve hypoxia, usually reported in diabetes (Ziegler and Gries, 1997; Vallianou *et al.*, 2009; Garcia-Alcala *et al.*, 2015). However, there is paucity of information on the ameliorative and protective effects of ALA either singly or in combination with other drugs in the treatment of canine babesiosis.

The present study, to the best of our knowledge is the first to report the use of ALA singly or in combination with imidocarb dipropionate in the treatment of canine babesiosis. The results obtained may improve our understanding on the pathogenesis, the clinical manifestations and the role of oxidative stress in canine babesiosis. It may also shed more light on the ameliorative role of ALA singly or in combination with imidocarb dipropionate on clinical manifestations, vital parameters and haemato-biochemical changes in dogs with babesiosis. Therefore, it was hypothesised that the use of ALA, as a potent antioxidant may

ameliorate the adverse effects caused by babesiosis, if found to be beneficial, ALA may be inculcated into the treatment protocol for canine babesiosis.

Most of the drugs used for the treatment of canine babesiosis have side-effects and often do not prevent relapse of the infection. There is the need to investigate the possible oxidant mechanism underlying the pathogenesis of canine babesiosis. Results of such study may be beneficial in the elucidation of the role of oxidative stress in the pathogenesis, and, consequently, more efficient control of the disease. Oxidative stress approach to the chemotherapy of canine babesiosis may be of immense benefit in the reduction of huge economical losses, often associated with the disease. The approach may be of value in developing more efficient prophylactic and therapeutic interventions. ALA is a potent natural antioxidant that is capable of crossing the blood-brain barrier, exerting no adverse or toxic effects on the body (Shay *et al.,* 2009). There is a need to explore the possibility of using antioxidant agents that may potentiate or act synergistically with the conventional chemotherapeutic agents.

1.4 Aim of the Study

The aim of the study was to evaluate the therapeutic roles of ALA singly or in combination with imidocarb dipropionate on clinical and pathological changes in Nigerian indigenous dogs experimentally inoculated with *Babesia canis vogeli*.

1.5 Specific Objectives

The objectives of the study were to determine the:

i. genetic identity of the inoculated *Babesia canis vogeli* using polymerase chain reaction and its evolutionary relationship with available sequences in the GenBank.

ii. effects of *Babesia canis vogeli*, treatment with imidocarb dipropionate and or ALA on clinical manifestations and vital parameters in Nigerian indigenous dogs with babesiosis.

iii. effects of *Babesia canis vogeli*, treatment with imidocarb dipropionate and or ALA on haematological changes and erythrocyte osmotic fragility in Nigerian indigenous dogs with babesiosis.

iv. effects of *Babesia canis vogeli*, treatment with imidocarb dipropionate and or ALA on serum biochemical parameters, serum proteins, liver transaminases and serum trace elements in Nigerian indigenous dogs experimentally inoculated with *Babesia canis vogeli*.

v. effects of *Babesia canis vogeli*, treatment with imidocarb dipropionate and or ALA on erythrocyte malondialdehyde concentration, platelet glutathione peroxidase and serum superoxide dismutase in Nigerian indigenous dogs.

vi. effects of *Babesia canis vogeli*, treatment with imidocarb dipropionate and/or ALA on tumour necrosis factor-alpha in Nigerian indigenous dogs.

1.6 Research Questions

i. Is molecular detection of *Babesia canis vogeli* significant in dogs with low parasitaemia?

ii. Does *Babesia canis vogeli*, treatments with imidocarb dipropionate and or ALA exert any significant effect on clinical manifestations and vital parameters in canine babesiosis?

iii. Does *Babesia canis vogeli*, treatments with imidocarb dipropionate and or ALA exert any significant effect on haematological changes and erythrocyte osmotic fragility in canine babesiosis?

iv. Does *Babesia canis vogeli*, treatments with imidocarb dipropionate and or ALA exert any significant effect on serum biochemical parameters, serum proteins, liver transaminases and serum trace elements in dogs experimentally inoculated with *Babesia canis vogeli*?

v. Does *Babesia canis vogeli*, treatments with imidocarb dipropionate and or ALA exert any significant effect on erythrocyte malondialdehyde concentration, platelet glutathione peroxidase and serum superoxide dismutase in Nigerian indigenous dogs?

vi. Does *Babesia canis vogeli*, treatments with imidocarb dipropionate and or ALA exert any significant effect on tumour necrosis factor-alpha in Nigerian indigenous dogs?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Babesiosis

Babesiosis is a disease caused by an intra-erythrocytic Apicomplexan protozoan, belonging to the genus *Babesia* (Singh *et al.*, 2014). It infects domestic animals, including sheep, goats, pigs, horses, cattle (Ziapour *et al.*, 2011); and wildlife, including mongoose, hyenas, kangaroos, weasels, polecab, elephants, raccoons, foxes and deer (Mosqueda *et al.*, 2012; Dawood *et al.*, 2013; Mahmoud *et al.*, 2015; Alvarado-Rybak*et al.*, 2016). The disease causes huge economic losses in the livestock industry all over the world (Lozano, 2011). None of the *Babesia* species that affect dogs and/or cats is considered to be of zoonotic importance (Homer *et al.*, 2000). Moreover, there is lack of evidence that *Babesia* spp., known to be zoonotic, actually has the capacity to infect dogs. Human babesiosis is a rare disease and primarily caused by two species of *Babesia: Babesia divergens,* a parasite of cattle in Europe; and *Babesia microti* that infects small rodents in the United States (Homer *et al.*, 2000). Variants of these two zoonotic species in Europe include a *B. divergens*-like species, *Babesia* spp. (also called *B. venatorum*) and a *B. microti-like species,* which is only sporadically reported (Yabsley and Shock, 2012). Infection by *B. divergens* after transmission by *Ixodes ricinus* (Morch *et al.*, 2015) results in severe clinical disease, especially in immune-compromised patients, undergoing chemotherapy, splenectomised, or human immunodeficiency virus positive. In immune-competent individuals, it presents as a mild or sub-clinical infection (Yabsley and Shock, 2012). There are over 100 *Babesia* spp. reported in vertebrate hosts (Koster *et al.*, 2015), including humans, that can be infected with *Babesia* spp.

Canine babesiosis causes high morbidity and mortality (Goddard *et al.,* 2013) with clinical manifestations and pathogenesis similar to malaria in humans (Koster *et al.,* 2015). It is a tick-borne haemo-protozoan disease with a worldwide distribution (Sudhakara *et al.,* 2016). *Babesia* species are often referred to as piroplasms, a collective term for phenotypically similar protozoan parasites that use mammalian RBCs in their life cycle. Piroplasms of domestic animals comprise two main genera, *Babesia* and *Theileria*. Infection of dogs by the intra-erythrocytic parasites results in a wide range of clinical manifestations; from sub-clinical to severe infection. Severe infection is characterised by fever, pale ocular mucous membrane, jaundice, splenomegaly, weakness and lethargy due to intravascular and extravascular haemolysis. Other manifestations of the disease include hypoxic injury, systemic inflammation, thrombocytopenia and pigmenturia (Irwin, 2009).

2.1.1 Different *Babesia* spp. and geographical location

The taxonomy of the protozoan (piroplasm merozoites) was determined by the morphology of the parasite within the RBCs. The protozoa is classified as either large (for example, *Babesia canis canis*) or small form (for example, *Babesia gibsoni*); and by microscopically evaluating the blood smear. On the basis of the genetic data, vector specificity, geographic distributions and variations in clinical signs and pathogenicity, the large *Babesia* spp., previously considered to be *B. canis canis,* currently include *B. canis canis, B. canis rossi and B. canis vogeli* as distinct sub-species (Schnittger *et al.,* 2012; Schaarschmidt *et al.,* 2013; Paulauskas *et al.,* 2014; Solano-Gallego *et al.,* 2016). Their identical morphology initially led *B. rossi* and *B. vogeli* to be thought of as sub-species of *B. canis,* although significant differences in their clinical presentation, geographical distribution and vector specificity (Table 2.1) have now led to their being considered otherwise (Solano-Gallego *et al.,* 2016). There are at least three genetically distinct small piroplasms that can cause

disease in dogs: *Babesia gibsoni, B. conradae and B. microti*-like (Ayoob*et al.,* 2010; Baneth*et al.,* 2015; Nalubamba *et al.,* 2015). In Africa, *B.canis canis, B. canis rossi, B. canis vogeli*, and *B. gibsoni* are reported, with no report of the micro *Babesiae* (Ayoob*et al.,* 2010; Solano-Gallego and Baneth, 2011). *Babesia rossi* is only reported in Africa (Solano-Gallego and Baneth, 2011). There also exists one yet unnamed *Babesia* species, reported in North America (Ayoob*et al.,* 2010; Solano-Gallego and Baneth, 2011).

2.1.2 Diversity among *Babesia canis* strains

2.1.2.1 Virulence differences

Virulence of *Babesia* strain is variable and it depends on the age, species and geographic location of the parasite (Halos *et al.,* 2014). In bovine babesiosis, host, parasite and vector co-evolve and the younger animals develop immunity after infection without showing clinical manifestations (Penzhorn, 2011) which is different in canine babesiosis. Strain diversity in *Babesia canis* could explain the variable clinical picture observed in affected dogs in France (Bourdoiseau, 2006). Direct evidence of the existence of strains of *B. canis canis* with variable virulence has recently been reported from Poland (Lyp *et al.,* 2016). Based on differences in part of the 18S ribosomal RNA gene, isolates of *B. canis canis* could be separated into three genetically different groups: A, B and C (Carcy *et al.,* 2015; Lyp *et al.,* 2015). Thrombocytopenia, one of the first haematological changes of natural and experimental *B. canis canis* infection (Cardoso *et al.,* 2008; Schetters *et al.,* 2009), significantly differs between the two genetic groups (Adaszek *et al.,* 2009). In addition, there is a strong correlation between the extent of thrombocytopenia and increases in rectal temperature (RT), accelerated pulse rate (PR) and discolouration of urine. The data support the hypothesis that virulence is indeed different between strains of *B. canis*. *Babesia canis rossi* has been reported to be the most virulent of the three sub-species of *B. c. canis*,

especially in South Africa (Penzhorn, 2011), and this might be as a result of the *Babesia rossi* erythrocyte membrane antigen 1 (*Br*EMA 1) gene (Adamu *et al.,* 2014; Nalubamba *et al.,* 2015).

2.1.2.2 Genetic diversity

With the development of molecular biological tools, it is possible to analyse the diversity of *B. canis* strains at the genetic level. Analysis of more than 90 isolates from France revealed that, based on diversity of the Bc28 gene, three genetically distinct groups could be defined, Bc28-A, Bc28-B (predominant) and Bc28-3403 (Carcy *et al.,* 2015; Mitkova *et al.,* 2017). Similar analysis of over 200 isolates from Central Europe revealed that only genetic groups, Bc28-A and Bc28-B are prevalent, with a slight predominance of Bc28-A. The analysis does not allow a correlation with virulence to be established; however, a correlation with functional protective immunity in vaccination challenge studies is apparent (Rene *et al.,* 2012).

Table 2.1: Epidemiological distribution, clinical manifestations and prognosis of *Babesia* spp. in dogs

Species	Vector	Size (µm)	Geographic location	Host Age	Onset of Infection	Clinical Manifestations	Virulence	Prognosis
Large								
Babesia canis	*Dermacentor reticularis*	2.5 x 4.5	Europe	Young, adult, hunting dogs	Acute	Lymphadenomegaly, hypotension, nonregenerative, normochromic normocytic anaemia, regenerative anaemia (uncommon) liver enzymes elevation, prerenal and renal azotaemia	Moderate to severe	Good to poor
Babesia vogeli	*Rhipicephalus sanguineous*	2.5 x 4.5	World-wide	Puppies or adult dogs	Acute and chronic	Regenerative immune-mediated haemolytic anaemia, nonregenerative anaemia, leukocytosis and leukopaenia	Mild to moderate	Good
Babesia rossi	*Haemaphysalis elliptica*	2.5 x 4.5	Sub-Saharan Africa,South Africa	Adult	Severe	Anorexia, splenomegaly, tarchycardia, anaemia, haemoglobinuria and bilirubinuria	Severe	Poor
Small								
Babesia gibsoni	*R. sanguineous H. longicornis*	1 x 3	Asia	Fighting dogs	Acute and chronic	Splenomegaly, small bowel diarrhoea, emaciation, elevated liver enzymes, mild to severe regenerative immune-mediated haemolytic anaemia, neutropaenia and leukocytosis.	Moderate to severe	Guarded to poor
Babesia microti-like	*Ixodes hexagonis I. canisuga*	1 x 2.5		Young, adult, guard/hunting dogs	Acute and chronic	Hyperglobinaemia, proteinuria, azotaemia	Moderate to severe	Guarded to poor

2.1.2.3 Antigenic variation

A further and distinct mechanism for the generation of antigenic diversity takes place via a dynamic genetic process operating at the clonal level. This phenomenon has been described for several protozoan parasites, including *B. bovis* (Allred and Al Khedery, 2004); where antigenic molecules are not expressed at the merozoite surface, but are instead expressed at the surface of the infected RBCs. The molecules may therefore play a role in the specific adhesion of infected RBCs to the capillary endothelium of particular organs, a process termed sequestration. It is believed that this process plays a role in sustaining low-level chronic infection in immune animals, which helps to maintain immunity (Allred and Al Khedery 2006). Although antigenic variation has not been shown for *B. canis*, low-level chronic infection occurs in dogs experimentally infected with *B. canis* or *B. vogeli* parasites (Vercammen *et al.,* 1995; Brandao *et al.,* 2003). In the latter in the dog, it is prolonged compared to those dogs treated after initial infection to clear the parasites (Brandao *et al.,* 2003). In the field, dogs are faced with several antigenically-diverse *B. canis canis* parasite families of variable virulence, and these have evolved mechanisms that allow survival in (partially) immune dogs (Solano-Gallego *et al.,* 2016).

2.2 Clinical Classification of Canine Babesiosis

The clinical manifestations of babesiosis are classified into three stages as hyper-acute, acute, and chronic or sub-clinical (Niwetpathomwat, 2006). Infected dogs may present different clinical manifestations, such as anorexia, fever, lethargy, acute haemolytic anaemia, thrombocytopenia, lymphadenopathy, and splenomegaly (Vishnurahav *et al.*, 2015). Others include haemoglobinuria and nervous signs (muscle twitching, incoordination and convulsion). The various clinical manifestations depend on the severity of the disease in infected animals which include anaemia, thrombocytopenia, leucocyte

abnormalities, increased activities of liver enzymes, and hyperbilirubinaemia (Solano-Gallego *et al.,* 2016). Hypokalaemia, hyperglobulinaemia, azotaemia, metabolic acidosis, and abnormalities of urinalysis may be observed in some severely affected dogs (Irwin and Hutchinson 1991; Niwetpathomwa, 2006). The clinical manifestations and outcome of canine babesiosis depend upon the infecting species and host immunity. The incubation period is around 10 - 28 days which means the disease manifests after the vector tick has fed and detached from its host, a process which is usually completed within a week (Sanchez-Vizcaino *et al.,* 2016). Most infections are reported in spring and/or summer and between May and July in Nigeria (Egege *et al.,* 2008), but the disease occurs throughout the year (Egege *et al.,* 2008). It is characterised by fever, lethargy, and varying degrees of haemolytic anaemia with associated signs (Koster *et al.,* 2015). Following the acute phase, most dogs become chronically infected with no or only poorly characterised signs. To a large extent, the manifestations and outcome of infection depend on the *Babesia* spp. involved (Schoeman, 2009). Canine babesiosis is categorised into three basic types, based on the onset, duration and severity of the disease, host infection, such as age and immunological response generated against the parasite (Andoni *et al.,* 2012; Torbica *et al.,* 2013).

2.2.1 Hyper-acute babesiosis

The hyper-acute stage is characterised by hypotensive shock, hypoxia, extensive tissue damage, and vascular stasis (Taboada, 1998). Usually, the infected dog is presented in shock or coma or dead, with history of less than a day anorexia and lethargy. It is common in puppies as young as four weeks (Abdullahi *et al.,* 1990).

2.2.2 Acute babesiosis

It is common in Asia, Southern Europe and Africa. It has been reported in Nigeria (Abdullahi *et al.,* 1990) in dogs of one to six-month-old. The incubation period of the acute form is 4 - 6 days. Within 3 - 4 days after onset, haemolytic syndrome is obvious with pale mucous membrane and jaundice (Ramona, 2014). This form of babesiosis is characterised by lethargy, anorexia, fever, lypmphadenopathy, haemolytic anaemia, haemoglobinuria, splenomegaly, emaciation and icterus (Schoeman, 2009). The anaemia is usually regenerative and dogs are often presented with anorexia and lethargy of 2 to 3 days. Haematuria and vomiting are common (Ramona, 2014).

2.2.3 Chronic/sub-clinical babesiosis

This occurs in adult dogs and it is characterised with intermittent fever, decreased appetite, weight loss, mild anaemia, depression, dehydraton, hypotension, lethargy, myalgia, muscle tremors, abdominal pain, bilirubinuria, pulmonary and renal dysfunctions (Abdel-Rhman *et al.*, 2015). Parasitaemia is difficult to identify in the peripheral blood (Ramona, 2014).

2.3 Pathophysiology of Babesiosis

Babesia species cause systemic hypotension, a compensatory fluid shift from the interstitial to the intravascular compartment in response to the immediate decrease in packed cell volume (PCV) and increase in plasma volume (Schetters *et al.,* 1998). Canine babesiosis is clinically classified into uncomplicated and complicated forms (Mrljak *et al.,* 2014; Omobowale *et al.,* 2017). Dogs with uncomplicated babesiosis are usually presented with signs relating to acute haemolysis, pale mucous membrane, fever, anorexia, depression, splenomegaly (Mrljak *et al.,* 2014). The clinical manifestations of complicated form are variable and related to the host's inflammatory responses, which is referred to as systemic

inflammatory response (SIR), eventually leading to pathologic changes in a number of organs causing multiple organ dysfunction syndrome (MODS) (Omobowale *et al.,* 2017).

2.3.1 Uncomplicated and complicated babesiosis

Subjects infected with *Babesia* parasites develop anaemia. The mechanism that leads to the anaemic state is complex and involves RBC depletion as a result of parasite multiplication, haemodilution, localisation in the spleen, (autoimmune) haemolysis, erythro-phagocytosis and impaired erythropoiesis (Jacobson, 2006). Anaemia may lead to tissue anoxia, considered to be the hall-mark of babesiosis (Lobetti, 1998). There are indications that oxidative stress and lipid peroxidation play a role in the pathogenesis of anaemia in some protozoan diseases (Rezaei and Dalir-Naghadeh, 2006), including babesiosis (Esmaeilnejad *et al.,* 2014), hepatozoonosis (Sarma *et al.,* 2015), trypanosomosis (Odeyemi *et al.,* 2015), theileriois (Verma and Singh, 2016).

Excess lipid peroxidation in biological membranes compromises structural integrity of cells, with loss of fluidity, decrease in membrane potential and increased permeability to ions (Gutteridge, 1995). These changes lead to rupture of the membrane and release of cell contents (Halliwell and Chirico, 1993). Scala *et al.* (2016) showed that lipid peroxidation causes an accumulation of oxidative ions in RBCs that causes their lyses. Many parasites including protozoa are sensitive to oxidative stress (Singh *et al.,* 2017). Sensitivity to oxidative stress has been reported in malaria (Rockett *et al.,* 1991), hepatozoonosis (Kiral *et al.,* 2005), theileriosis (Visser *et al.,* 1995) and bovine babesiosis (Stich *et al.,* 1998). ROS and RNS are powerful oxidants and nitrating species that can inactivate enzymes and initiate lipid peroxidation and nitration, which in turn lead to free-radical chain reactions that further damage membranes, nucleic acids and proteins (Muller *et al.,* 2003). The

processes can ultimately lead to parasite death (Rockett *et al.,* 1991; Kiral *et al.,* 2005). As a defence mechanism, parasites synthesise compounds and antioxidant enzymes that directly neutralise ROS and RNS. Over recent years, several antioxidant and detoxification systems of parasitic protozoa have been identified and their roles in parasite survival have been investigated (Muller *et al.,* 2003). These so-called 'redox proteins' were shown to be essential for the survival of protozoan parasites (Muller *et al.,* 2003).

Furthermore, oxidative stress induces organ-injury and negatively affects overall survival of animals (Crimi *et al.,* 2006; Kucukkurt *et al.,* 2014). Malondialdehyde (MDA) is an end-product of polyunsaturated fatty acid oxygenation, and is a reliable and commonly used biomarker for assessing lipid peroxidation (Moore and Roberts, 1998). Serum MDA levels are elevated significantly in canine babesiosis (Crnogaj *et al.,* 2010). Products of oxidative stress may, thus, contribute to protective immune responses against the parasite, if produced in optimal amounts (Visser *et al.,* 1995; Rahal *et al.,* 2014); but may also contribute to pathogenesis when produced in excess (Stich *et al.,* 1998; Salem *et al.,* 2016). Therefore, the important factor determining favourable outcome in babesiosis is apparently the balance between oxidant and antioxidant processes (Baldissera *et al.,* 2015).

Different levels of parasitaemia have been reported in numerous studies of canine babesiosis. Anaemia in canine babesiosis is not proportional to parasitaemia (Narurkar *et al.,* 2017). The established fact that the quantity of destroyed RBCs is much higher than the degree of parasitaemia suggests that destruction of both parasitised and unparasitised RBCs occur (Murase and Maede, 1990). In one study, all patients showed a level of parasitaemia of below 1% (Jacobson and Lobetti, 2005); while another study (Bohm *et al.,* 2006) reported that higher levels of parasitaemia correlate with worse clinical scores, and that the

degree of parasitaemia was significantly higher in the dogs that died. In an experimental canine babesiosis caused by *B. canis canis,* all dogs developed low-grade parasitaemia (less than 1%) (Schetters *et al.,* 2009). In studies of canine babesiosis in Croatia, all patients with uncomplicated babesiosis had a level of parasitaemia below 1% (Eichenberger *et al.,* 2016). In another study of septic shock due to babesiosis in Croatia, seven of 10 dogs had a level of parasitaemia above 1%, whereas three had a level below 1% (Matijatko *et al.,* 2009). Since all the dogs in this study died, it is obvious that a low level of parasitaemia may not guarantee a favourable outcome, but a level of parasitaemia greater than 1% is associated with poor outcome.

One of the proposed mechanisms underlying the development of septic shock in babesiosis and, subsequently, increasing the risk of poor outcome, is related to the parasite itself. The association between a higher level of parasitaemia and shock raises the question of whether the two are causally related. In experimental *B. canis canis* infection, the onset of the acute phase reaction appears to be related to the infectious dose, although the acute phase response is ultimately triggered in all cases, irrespective of dose (Schetters *et al.,* 2009). The arguments for the inflammatory response are supported by the fact that acute-phase protein production increases in canine babesiosis and may serve as a predictive marker for disease risk and for monitoring the response to treatment (Matijatko *et al.,* 2007). Nevertheless, the concentration of C-reactive protein (CRP) does not show prognostic value because it is not associated with favourable outcome in babesiosis caused by *B.canis rossi* (Koster *et al.,* 2009). The timing of the different triggers may lead to different outcomes: high infectious doses trigger an early inflammatory response that could destabilise the system (Matijatko *et al.,* 2012). Severe parasitaemia is unlikely to be the sole trigger of circulatory collapse in canine babesiosis. This is particularly true because some dogs with

low levels of parasitaemia are known to develop shock (Bohm *et al.,* 2006; Matijatko *et al.,* 2009; Schetters *et al.,* 2009); suggesting that this response is triggered by soluble parasite antigens (SPA) released into the plasma during infection (Schetters, 2005; Schetters *et al.,* 2009). Abnormalities reported in complicated canine babesiosis cases include hepatopathy, acute kidney injury, cerebral babesiosis, acute respiratory distress syndrome, relative haemoconcentration ("red biliary"), pancreatitis and rhabdomyolysis, and myocardial dysfunction (Koster *et al.,* 2015).

2.4 Canine Babesiosis

For a long time, it was widely accepted that babesiosis caused by *B. canis rossi* is a disease different from babesiosis caused by *B. canis canis*. Most importantly with *B. canis rossi* infections, there is a higher risk of developing complications and a significantly higher rate of mortality (Koster *et al.,* 2015). By comparison, infection with *B. canis canis* was considered to cause a predominantly mild disease with high sero-prevalence ranges (20 - 85%) in endemic areas (Beck *et al.,* 2009), and low rate of clinical disease (Martinod *et al.,* 1986). The results of studies of babesiosis caused by *B. canis canis* throughout Europe demonstrated a wide range of mortality rates, varying from 1.5 to 20%. The highest mortality rates are found in Hungary (20%) (Mathe *et al.,* 2006a), the Netherlands (17%) (Matjila *et al.,* 2005) and Croatia (11–13.9%) (Allred and Al-Khedery, 2004), 9% in Portugal (Cardoso *et al.,* 2010); and the lowest rate was recorded in France (1.5%) (Martinod *et al.,* 1986). In addition, reported mortality rates for dogs with babesiosis in Portugal should be interpreted in the light of the fact that two of four dogs that died were euthanised without treatment, the third was infected with *B. canis vogeli, Ehrlichia canis* and *Leishmania infantum*, and the fourth was infected with *B. canis canis* and *Leishmania infantum*. From these data, the actual mortality rate in Portugal for *B. canis* infection

without co-infection was low. Therefore, *B. canis canis* disease in both the Netherlands and Portugal can be considered to be mild.

By contrast, the clinical manifestations, complications and mortality rate of *B. canis canis* infection in Hungary and Croatia are more similar to those of South African canine babesiosis, caused by *B. canis rossi*, than to *B. canis canis* infections in other European countries (Mathe *et al.,* 2006b). Overall, it seems that canine babesiosis caused by *B. canis canis* in Europe is not a single disease, but rather has at least two clinically different disease manifestations; one mild, in which multiple organ failure is absent; and a second that is severe and generally complicated by MODS and hypotension. The reason for this difference might lie in the parasite itself, the host, or in host-parasite interplay. Regarding the parasite itself, it has been demonstrated that different *Babesia* species, sub-species or isolates can produce disease of markedly different severity (Reyers *et al.,* 1998). Moreover, significant differences in disease outcome and prognosis have been observed following infection with different strains of a single species of *Babesia (B. canis rossi)* (Matjila, 2009). Regarding the host response, in some human patients with malaria, organ system dysfunction may develop and evolve even after the parasites have been eradicated from the blood by specific therapy (Udwadia, 2003). This observation supports the hypothesis that inflammatory mediators are involved because immuno-inflammatory processes set in motion by fulminant infection may at times be self-perpetuating, even when the parasites have been eliminated by treatment. It is clinically important to understand that the pro-inflammatory state of the acute phase response also initiates anti-inflammatory mediators involved in compensatory anti-inflammatory response syndrome (CARS). The extent of the pro- and anti-inflammatory events should be balanced and proportional to the insult (Matijatko *et al.,* 2012).

Some factors, including constitutional, genetic, and/or environmental, may generate an inadequate reaction, over-reaction and under-reaction. The balance between systemic inflammatory response syndrome (SIRS) and CARS plays a crucial role in resolving the infection, but the pro- and anti-inflammatory mechanisms are often deregulated for reasons that are not entirely understood (Matijatko *et al.,* 2012). In summary, the differences in clinical manifestations of babesiosis appear to be the result of the interplay of parasite-host interactions. If the animal responds quickly with robust inflammatory cytokine production, the parasite may be quickly controlled; but if the pro-inflammatory response is inadequate, then surviving parasites will continue to trigger responses, resulting in the clinical picture of MODS. The clinical picture is a reflection of the failure of the pro-inflammatory cytokine response to be switched off in time, leading to a sustained damage to host tissues and organs. This could explain why vaccination with SPA is effective: the secondary antibody response could limit further the triggering of the pro-inflammatory response, initiated by SPA (Schetters, 2005).

2.4.1 Infections with Different Species of *Babesia*

2.4.1.1 Infection with Babesia canis canis

B. canis canis mostly causes mild to severe infection with often low parasitaemia which does not necessarily correlate with the severity of clinical disease (Uilenberg *et al.*, 1989). It causes the acute form of babesiosis which is characterised with fever, anorexia, lethargy and dehydration. There is mild to severe thrombocytopaenia, mild to moderate normocytic normochromic non-regenerative anaemia, haemolysis and neutropenia. Haemoglobinuria has been reported in naturally infected dogs (Solano-Gallego and Baneth, 2011), which usually occurs during autum or spring due to the favourable condition of tick vector activity (Solano-Gallego *et al.,* 2008). Experimental *B. canis canis* infection can result to transient

low parasitaemia (1 - 2%), decreased PCV, thrombocytopenia, hypotension and increase in the activated partial thromboplastin time (APTT) (Solano-Gallego and Baneth, 2011).

2.4.1.2 Infection with Babesia canis vogeli

In sub-Saharan Africa, *Babesia canis vogeli* was first reported from clinically healthy dogs in South Africa (Matjila *et al.,* 2004), and in stray cats of metropolitan Bangkok, Thailand (Simking *et al.,* 2010). Its presence had probably been overlooked because of the preponderance of infections of the highly virulent *B. canis rossi* (Matjila *et al.,* 2008). *Babesia vogeli* has also been reported from Slovenia (Duh *et al.,* 2004), Sudan (Oyamada *et al.,* 2005), Portugal (Cardoso *et al.,* 2008), Cape Verde (Götsch *et al.,* 2009), Columbia (Vargas-Hernandez *et al.,* 2012), Southern France (Rene *et al.,* 2012), Romania (Ionita *et al.,* 2012), Nigeria (Sasaki *et al.,* 2007; Kamani *et al.,* 2013; Adamu *et al.,* 2014), Brazil (Duarte *et al.,* 2011; Araujo *et al.,* 2015; Silva *et al.,* 2016), Philipines (Ybanez *et al.,* 2016) and Central Namibia (Penzhorn *et al.,* 2016). In Zimbabwe, *B. canis vogeli* has been reported from captive lions (*Panthera leo*), wild cats (*Felis lybica*) and servals (*Leptailurus serval*) and could presumably also occur in dogs (Kelly *et al.,* 2014; Solano-Gallego *et al.,* 2016). This is the least virulent species, although it causes clinical disease ranging from severe to fatal haemolytic anaemia in puppies (Solano-Gallego and Baneth, 2011). It usually causes sub-clinical to mild or moderate infections, with a low parasitaemia in adult dogs. The lack of virulence might be because of its long association with the domestic dog (Shakespeare, 1995; Penzhorn, 2011).

2.4.1.3 Infection with Babesia canis rossi

Babesia rossi was first described from a side-striped jackal (*Canis adustrus*) and black-backed jackals (*Canis mesomelas*) in Kenya (Penzhorn, 2011; Penzhorn *et al.,* 2017). It

causes the most severe infection among the large babesial species that infect dogs and most prevalent in summer (Jacobson, 2006). Clinical manifestations observed may be categorised in two: uncomplicated with a relatively uncompromised blood circulatory system (prognosis is usually good) or complicated with a clinically compromised circulation, usually with poor prognosis. Prognosis is favourable if there is no presence of multiple organ dysfunctions. Mortality rate in dogs with complicated babesiosis is about 15%, irrespective of the nature of the treatment administered. The disease is characterised by pale mucous membrane, depression, tarchycardia, high parasitaemia, tarchypnoea, anorexia, weakness, and coma at presentation, hypoglycaemia and high serum lactate as well as cerebral and renal involvement (Penzhorn, 2011). It is the most common cause of virulent babesiosis in South Africa (Liebenberg *et al.*, 2013; Penzhorn *et al.*, 2016), though it has been reported in Nigeria (Sasaki *et al.*, 2007; Kamani *et al.*, 2013; Adamu *et al.*, 2014; Adebayo *et al.*, 2016).

A polymorphic phosphoprotein localised on the cytoplasmic surface of *B. rossi*-infected red blood cells has recently been characterised and named *B. rossi* erythrocyte membrane antigen 1 (*Br*EMA 1). This protein is suspected as the virulence factor in *B. rossi* canine babesiosis (Matjila et al., 2009); which is absent in the less pathogenic species of *Babesia*, including *B. canis canis* and *B. canis vogeli* (Solano-Gallego and Baneth, 2011). In some dogs, not all the clinical changes can be attributed to the haemolytic anaemia and hypoxia; such dogs are regarded as having complicated canine babesiosis, a description loosely based on the World Health Organisation (WHO) classification for malaria (Jacobson and Clark, 1994). In addition to the *Babesia* induced haemolysis, dogs with complicated babesiosis have immune-mediated haemolytic anaemia (IMHA) and/or signs from inflammatory reactions (Reyers *et al.*, 1998).Dogs with complicated forms of babesiosis are

extremely challenging to treat with a mortality rate reportedly to be as high as 45% in a study. There is a higher mortality rate (29%) in dogs that develop a severe inflammatory reaction than in those that develop severe anaemia (8%), indicating the severity of the inflammatory response (Reyers *et al.,* 1998).

2.4.1.4 Infection with Babesia gibsoni

Most infections cause mild manifestations in the acute phase, but some *B. gibsoni* infections can cause severe anaemia and be mis-diagnosed as immune-mediated haemolytic anaemia (IMHA); either due to the parasite not being visible on blood films due to low parasitaemia, or due to low level of suspicion in non-endemic areas (Conrad *et al.,* 1991).*B. gibsoni* can cause hyper-acute, acute, and chronic infections. Hyper-acute infections are rare and primarily occur in puppies, resulting in rapid death. These infections are presumed to be maternally acquired. Acute *B. gibsoni* infections are typically associated with fever, lethargy, thrombocytopenia, and anaemia (Sunaga *et al.,* 2014; Brown *et al.,* 2015). Chronic infections may be completely asymptomatic or may be characterised by intermittent fever, lethargy, and weight loss. Most dogs are depressed, have a history of anorexia, and are diagnosed with regenerative anaemia (Farwell *et al.,* 1982).Many dogs are Coombs' test positive. Despite the thrombocytopenia, coagulation profiles are normal with no clinical signs of haemorrhage (Birkenheuer *et al.,* 1999). A case of protein-losing nephropathy (membrano-proliferative glomerulo-nephritis and immune complex deposition) has been described in a Labrador retriever that was PCR-positive for *B. gibsoni* and resolved with elimination of the infection (Slade *et al.,* 2011). Cutaneous vasculitis secondary to *B. gibsoni* infection occurred in a Satsuma dog, characterised by generalised alopecia, ear-tip papules and erosions, and necrosis of the skin of the fore-limb (Koster *et al.,* 2015). The skin changes were due to immune complex adherence to the walls of blood

vessels and peri-vascular pathology (Tasaki *et al.,* 2013). Manifestations in chronic infections are common, including low-grade fever, pallor, splenomegaly, and lymphadenopathy.

2.5 Diagnosis of Babesiosis

The basic methods used for diagnosing babesiosis are based mainly on observation of clinical manifestations, microscopic examination of blood smears stained with Giemsa, and serological analysis (Slodki *et al.,* 2011), with each of the methods having its limitations. However, other newer methods are necessary, including deoxyribonucleic acid (DNA) detection and isolation using polymerase chain reaction technique.

2.5.1 Microscopic examination

Microscopy remains the simplest and most accessible diagnostic test for most veterinarians. During acute infections, microscopy is reasonably sensitive for detecting intra-erythrocytic parasites in Giemsa- or Wright's-stained blood smears. Differentiation between large and small Piroplasms are also relatively simple. Moreover, microscopy is still the only viable option available to veterinarians in many parts of the developing world, where babesiosis is endemic. With large *Babesia* species at least, sampling from capillary beds (ear tip and toe nail) or examination of cells from beneath the buffy coat of a haematocrit tube may improve the probability of identifying the parasites (Matijatko *et al.,* 2009; Silva, 2016).

2.5.1.1 Thin and thick blood smear

Thin blood smears are the first method used to detect *Babesia* parasites in dogs with clinical manifestations and it is an effective diagnostic technique. The procedure is easy and inexpensive, but requires expertise to differentiate. It is reliable only if the level of

parasitaemia is high for identification (Mosqueda *et al.*, 2012). Limitations of this technique include misdiagnosis; especially in sub-clinical or chronic forms of babesiosis, where parasitaemia is low (Ybanez *et al.*, 2018). Some species, including *Babesia ovis* and *B. canis* that adhere to endothelial lining (Jacobson, 2006), are difficult to detect in peripheral blood taken from blood capillary from the ear or the skin of the tail (Mosquede *et al.*, 2012). Thick blood smears can be used to detect low levels of parasitaemia. It is advantageous because a large amount of erythrocytes is analysed in a reduced amount of space, and the probability of detecting infected RBCs is 10 times higer than in the thin smear (Mosquede *et al.*, 2012). The diagnosis of piroplasmosis in chronically-infected and carrier dogs, however, remains a significant challenge due to very low, often intermittent parasitaemias (Esmaeilnejad *et al.*, 2015). Failure to detect *Babesia* parasites in animals with haemolytic anaemia or thrombocytopenia has led to an incorrect diagnosis in documented cases, often when the clinical suspicion of babesiosis is also low. In low parasitaemia and due to the morphological similarity between species, the microscopic analysis of piroplasmosis is complicated and could lead to misdiagnosis (Irwin, 2009). Therefore, the use of molecular techniques is necessary to detect and identify *Babesia* species effectively (Paulauskas *et al.*, 2014).

2.5.2 Molecular diagnosis - conventional polymerase chain reaction

Polymerase chain reaction (PCR) has greatly increased the sensitivity and specificity of parasite detection and is well suited to epidemiological and phylogenetic studies, but molecular techniques for routine clinical diagnosis of babesiosis are restricted to relatively few laboratories worldwide. Ribosomal ribonucleic acid (RNA) genes 18S, 5.8S, 28S and the internal transcribed spacer (ITS) sequences have been used for conventional PCR, but some researchers have chosen other loci such as the p18/Bg Thrombospondin-related

adhesive proteins (TRAP) (Fukumoto *et al.,* 2001). The parasite morphology is a poor guide to speciation, therefore modifications of the PCR have been utilised to rapidly differentiate between piroplasm spp.; polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) and nested PCR have been reported to differentiate *B. vogeli* and *B. gibsoni* in Australia (Jefferies *et al.,* 2007), and between the large *Babesia* species (Zahler *et al.,* 1998) and *B. gibsoni* in endemic regions (Birkenheuer *et al.,* 2003). In a clinico-pathological study of large *Babesia* spp. infections in dogs in Italy using PCR-RFLP, *Babesia canis canis* was detected in 20.7% and *B. c. vogeli* in 6.71% dogs, although a distinct geographical difference in the incidence of these infections has been reported (Solano-Gallego *et al.,* 2008). Further refinement in primer design was reported recently to clearly separate amplicons of 342 bp, 546 bp, and 746 bp target fragments of *B. canis rossi, B. canis vogeli, B. canis canis,* respectively. Loop-mediated isothermal amplification (LAMP) was found to have advantages of speed and specificity for detecting *B. gibsoni* infections in Japan (Ikadai *et al.,* 2004) and reverse-line blot (RLB) hybridisation was applied in epidemiological studies of arthropod-borne haemopathogens of dogs and cats in Trinidad (Matsuu *et al.,* 2005) and dogs in Africa (Matjila *et al.,* 2008). Real-time PCR enables quantification of pathogen levels within blood and tissue samples; the amount of final PCR product may be used to deduce the starting number of target molecules and infer parasite levels within a host.

Quantitative PCR (qPCR) was used in an experimental infection of three dogs with *B. gibsoni* that concluded that the methodology may be adopted to determine vaccine or chemotherapeutic efficacy, or the elucidation of immunological responses (Matsuu *et al.,* 2005). A number of the PCR methods have been applied to filter-paper technologies, such as FTA cards (Whatman Bioscience) and IsoCode Stix (Schleicher and Scheull) for ease of

transport of samples to distant laboratories, and for epidemiological and other diagnostic studies (Jefferies *et al.,* 2007; Tani *et al.,* 2008). Long-term storage and archival qualities of this methodology have not been investigated (Tani *et al.,* 2008). The detection limit of light microscopy is approximately 0.001% parasitaemia, but PCR is capable of detecting parasite loads in the region of 50 organisms/mL (Camacho *et al.,* 2004) and 9 parasites/μL (Schetters *et al.,* 1995). Despite its extraordinary sensitivity, "False negative" results may occur in chronic babesiosis because of low parasitaemia (9 parasites/ μL) which is an important limitation, when screening potential carriers and other asymptomatic dogs such as blood donors.

The ability of PCR to detect infected dogs in such situations, with and without treatment, has been investigated (Florin-Christensen *et al.,* 2002; Allred and Al Khedery, 2006). In a study conducted by Allred and Al Khedery (2006), clinical parameters, haematology, serologic titre by indirect fluorescent antibody test (IFAT) and the presence of *Babesia* DNA was monitored on a daily basis after experimental infection. All dogs (n = 3) had a full clinical recovery, as evaluated by normal clinical manifestations, absence of splenic enlargement, a normal haemogram and absence of piroplasms on microscopic examination by 30 - 50 days after peak parasitaemia. During this period of clinical normality, *Babesia* DNA was inconsistently detected. This finding suggests a very low, fluctuating parasitaemia in these dogs, possibly analogous to chronic, asymptomatic natural infection. The ability of PCR to detect parasite DNA in chronically-infected animals may be improved by testing on more than one occasion, but the use of serology as an alternative, complementary diagnostic test is advisable in these situations (Matijatko *et al.,* 2012). Molecular methods present a higher sensitivity and specificity than the evaluation of

peripheral blood smears to detect babesial infection and morphological differentiation of similar species (Ionita *et al.,* 2012).

2.5.3 Serological testing - the indirect fluorescent antibody test and enzyme-linked immunosorbent assay

Indirect fluorescent antibody testing (IFAT) has been the most widely supported serological diagnostic test for canine babesiosis for the last 30 years and above (Anderson*et al.,* 1980; Levy *et al.,* 1987). However, poor specificity due to cross-reactions between *Babesia* spp. and with other Apicomplexan parasites, operator subjectivity and its inadequacy for large-scale screening have all been limiting factors (Esmaeilnejad *et al.,* 2015). Recent research in Japan has been directed towards finding specific immune-dominant *B. gibsoni* antigens for use in recombinant protein enzyme-linked immunosorbent assays (ELISA) and potential vaccine candidates in the future has identified numerous promising compounds (Irwin, 2009). Thrombospondin-related adhesive proteins (TRAPs) comprise a group of highly-conserved functional proteins, identified in Apicomplexan parasites, mooted to be associated with merozoite motility and invasion, and capable of inducing a host-antibody response (Zhou *et al.,* 2006).

An ELISA using recombinant BgTRAP is more sensitive than other ELISAs using recombinant antigens rBgP50, rBgSA1, and rBgP32 (Goo *et al.,* 2008), Konishi *et al.* (2008) utilised a BgTRAP ELISA to test 1,206 randomly selected non-fighting breed dogs in Japan to test for exposure to *B. gibsoni* (Konishi *et al.,* 2008). The authors reported higher infection rates in western Japan and concluded that having excluded fighting dogs, a history of tick (*Haemaphysalis longicornis*) exposure is a significant risk factor for anti-babesial antibody detection, thus demonstrating two distinct epidemiological patterns of *B. gibsoni* infection in Japan. A second major limitation of serological tests is their inability to

differentiate acute from chronic infections, and interpretation of a positive titre is somewhat problematic for clinicians working in regions that are endemic for babesiosis. Nevertheless, for *B. gibsoni* in the USA and Australia, where cases are sporadic, the IFAT is a useful tool for detection of infected dogs, especially if combined with PCR (Irwin, 2009). A new ELISA and PCR was developed to differentiate between acute *B. canis* infection and convalescent samples collected from six *B. canis* infected dogs at least 14 days post-treatment. The test which has a specificity of 86.4% detects infection 24 - 48 h before light microscopy, when the latter tests negative (Eichenberger *et al.*, 2017).

2.5.4 Diagnostic imaging

Hepatomegaly and splenomegaly are the most consistent findings on abdominal ultrasonography in dogs with *B. canis canis* (Fraga *et al.*, 2011; Sarma *et al.*, 2015). The largest study (n = 72) to describe ultrasonographic changes in dogs naturally infected with babesiosis, assumed to be *B. canis canis*, consistently found splenomegaly with diffuse, hypo-echoic, heterogeneity (Fraga *et al.*, 2011). Diffuse homogenous, increased cortical echogenicity and increased cortico-medullary definition have been attributed to the effects of haemoglobinuria, hypoxic damage, or immune-complex glomerulonephritis. Doppler characteristics were compared between dogs with babesiosis caused by *B. canis ro*ssi and dogs with severe anaemia; the renal interlobar artery resistivity index was significantly higher in the former group due to reduced diastolic velocities (Koma *et al.*, 2005). The study has convincingly demonstrated that the differences in the Doppler characteristics between the two groups are due to the increased resistivity index in the group of dogs with babesiosis; and thus, not solely due to anaemia.

In dogs infected with *B. canis canis*, the pulsatility index was significantly higher in those with severe uncomplicated babesiosis, and resistivity index was significantly higher in dogs with severe uncomplicated and complicated babesiosis (Koma *et al.,* 2005). The increases in pulsatility index and resistivity index could have been due to endothelin-induced vasoconstriction and part of the initiation phase of acute kidney injury, which was found in 75% of dogs with renal involvement and 80% of dogs that died from acute kidney injury. Ultrasonographic changes have been described in the pancreases of dogs infected with both *B. rossi* and *B. canis* that showed gastro-intestinal signs and abdominal pain (Mohr *et al.,* 2000). The changes are consistent with acute pancreatitis and include duodenal atony and peri-pancreatic fat hyper-echogenicity. Pulmonary perfusion studies using scintigraphy to evaluate the possibility of pulmonary thrombo-embolism in canine babesiosis failed to show pleural-based pulmonary wedge-shaped defects, consistent with the condition (Sweers *et al.,* 2008).

2.6 Clinical Biomarkers of Canine Babesiosis

2.6.1 Blood cellular components

Haematological (PCV, RBC and platelets) changes have been examined in several investigations in babesiosis (Furlanello *et al.,* 2005; Mathe *et al.,* 2006b; Schetters *et al.,* 2009). Following infection, there is usually a mild to moderately regenerative normocytic and normochromic anaemia because of the haemolysis (Scheepers *et al.,* 2011). Increased blood concentration indicates a poor prognosis, with very high mortality rates (Koster *et al.,* 2015) associated with elevated PCV in infected dogs. Neutrophil counts are usually normal to decreased levels, although left shifts are relatively common. Thrombocytopenia is a consistent finding, usually severe in the acute phase of infection. Platelet counts usually rise within a week of therapy. The severity and rapid recovery of the platelet counts has led to

the suggestion that immune-mediated mechanisms are involved in canine babesiosis (Arun *et al.,* 2017).

2.6.2 Coagulation profiles

Coagulopathies, both primary and secondary haemostasis abnormalities, have been described in both *B. canis canis* and *B. canis rossi* infections (Rafaj *et al.,* 2009; Goddard *et al.,* 2013; Rafaj *et al.,* 2013).Thrombin-antithrombin complexes are significantly higher and anti-thrombin activity significantly lower in *B. canis canis*-infected dogs than controls (Rafaj *et al.,* 2009).Goddard *et al.* (2013), demonstrated significant consumptive coagulopathy in non-survivors, compared to survivors in dogs with *B. canis rossi* infection (Koster *et al.,* 2015). Specifically, the median coagulation factor concentrations and protein-C activity are lower in non-survivors, and median D-dimer concentration was higher in the dogs that died. In uncomplicated *B. rossi* infections, a transient coagulopathy with abnormalities in prothrombin time, activated partial thromboplastin time, fibrinogen, D-dimer and thrombo-elastography were found in dogs, despite the lack of clinical haemorrhage (Liebenberg *et al.,* 2013). Infected dogs have significantly lower mean platelet count, higher fibrinogen concentrations, higher D-dimer value, prolonged activated partial thromboplastin time, and thrombo-elastography lysis values, consistent with clots more resilient to lysis, as compared to healthy controls. Increased concentrations of intercellular adhesion molecule, C-reactive protein, and fibrinogen and decreased concentration of von Willebrand factor occur in dogs in the acute stages of *B. canis* infection (Rafaj *et al.,* 2013).The mechanism of the haemostatic dysfunction observed in canine babesiosis may be the result of endothelial dysfunction and most likely contributes to RBC and, thus, parasite sequestration, which may be important in clinical outcome (Kules *et al.,* 2017).

2.6.3 Pro-inflammatory and anti-inflammatory cytokine profile

Infectious agents induce tissue macrophages, monocytes, mast cells, endothelial cells, platelets and other reactive cell elements to produce various pro-inflammatory mediators. Of these mediators, tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1) and IL-6 are the most widely known. Early events in the inflammatory response also induce the production of cortisol, another potent anti-inflammatory mediator. Through the combined action of these mechanisms, the acute phase response leads to resolution of the infection (Nystrom, 1998). A major role of pro-inflammatory cytokines has been reported in human (Shaio and Lin, 1998), bovine (Shoda *et al.,* 2000), equine (Hanafusa *et al.,* 1998) and canine babesiosis (Schetters *et al.,* 2009). However, the only pro-inflammatory cytokine that has been investigated in canine babesiosis is TNF-α, while the only anti-inflammatory mediator studied in canine babesiosis is cortisol. The principal limitations of the afore-mentioned studies are that only single pro-inflammatory and anti-inflammatory mediator were investigated using different samples, thus precluding the study of their possible interactions and correlations.

In canine babesiosis, there is an association between TNF-α concentration, clinical severity and parasitaemia (Vaughan-Scott *et al.,* 2004; Zygner *et al.,* 2014). Apparently, TNF-α plays a role in the production of other harmful substances including nitric oxide and ROS, and may mediate the interaction between parasitised RBCs and the blood vessel wall. Nitric oxide metabolites, nitrate and nitrite, collectively referred to as RNS are mediators of a multiple organ dysfunction syndrome, which occurs in complicated canine babesiosis, a putative sequela of SIRS (Jacobson *et al.,* 2002).In the acute virulent form of canine babesiosis, 87% of complicated cases fulfill the requirements for SIRS (Welzl *et al.,* 2001).However, the level of RNS has not been shown to correlate significantly with the

severity of illness, nor is it predictive of outcome (Crnogaj *et al.*, 2017). The concentration of acid glycoprotein, a positive acute phase protein, is considerably elevated in dogs, infected with *B. canis rossi*, but the level does not correlate with severity of disease or outcome (Lobetti *et al.*, 2000).The acute phase response, as measured by serum amyloid A and C-reactive protein concentrations, significantly decreases after successful treatment of *B. canis canis* infections, and together with platelet counts have been found to be the most sensitive parameters for monitoring therapeutic responses in infected dogs (Matijatko *et al.*, 2007).

2.6.4 Metabolic abnormalities

Hypoglycaemia is a common complication of canine babesiosis, associated with collapse, severe anaemia, icterus, seizures, and coma (Keller *et al.*, 2004).Reduced survival has reportedly been associated with hypoglycaemia (59.4 mg/dL) and hyperlactataemia at admission (Nel *et al.*, 2004).*Babesia*-infected dogs with hypoglycaemia have very high TNF-α values, which correlate with parasitaemia (Vaughan-Scott *et al.*, 2004).Neurological signs resulting from low glucose levels may be misdiagnosed as cerebral babesiosis (Keller, 2004).

2.6.5 Renal and liver function tests

Elevated serum urea levels are common in dogs with babesiosis, and this elevation is often disproportionate to the rise in creatinine. This is probably due to increased protein catabolism and urea production, resulting from gastro-intestinal haemorrhage or protein catabolism due to the febrile inflammatory illness (Reyers, 1992). In complicated babesiosis, the serum urea and creatinine levels may be proportionately increased; indicating decreased renal perfusion, possibly as a result of decreased blood pressure,

decreased myocardial function, and/or hypovolaemia. In a study conducted by Van Zyl (1995), it was showed that elevated creatinine was associated with increased risk of death, but it was not clear if the elevation was due to pre-renal azotaemia. Cystatin C (CST3) is a neuroendocrine basic polypeptide encoded by CST3 gene and has been proposed as a more sensitive marker than creatinine for reduced glomerular filtration rate due to renal insufficiency. However in a study, both the mean and median serum CST3 concentrations of dogs with babesiosis were within normal range (De Scally *et al.,* 2004).It has been shown that dogs with *B. canis rossi* infections had significantly higher concentrations of urinary IgG (uIgG), urinary C-reactive protein, and urinary retinol-binding protein, indicative of the presence of both glomerular and tubular dysfunction (Koster *et al.*, 2015). Although activities of urine enzymes (gamma-glutamyl transferase and alkaline phosphatase) are both an early and persistent indicator of renal tubular damage, in canine babesiosis, changes in urine enzyme activities appear to be variable and minimal, thus limiting their utility as diagnostic tests (Koster *et al.,* 2015). The elevation of urinary makers (uIgG, uCRP and uRBP) is superior to urinary specific gravity, serum urea and creatine concentration for early detection of renal dysfunction in dogs with babesiosis (Defauw *et al.*, 2012).

Elevated liver enzyme (alanine transferase, aspartate transaminase and alkaline phosphate) activities have been reported in dogs with babesiosis, suggestive of liver injury associated with hypoxic liver, which is seen in haemolytic anaemia in babesiosis (Zygner *et al.*, 2011). Chronic hepatic insufficiency occurs in babesiosis, which can lead to hypoalbuminaemia (Gonde *et al.*, 2014). Other clinical and haemato-biochemical findings in babesiosis include elevated unconjugated bilirubin, haemoglobinuria, haemolytic anaemia and icterus, reported to occur in advanced stages of canine babesiosis (Ajith *et al.*, 2017).

2.7 Alpha-Lipoic Acid

2.7.1 History of alpha-lipoic acid

Alpha- lipoic acid (ALA), also known as thioctic acid and 1,2 dithiolane-3-pentanoic acid (Figure 2.1), is a naturally occurring substance. It was discovered by Snell *et al.* (1937), but isolated by Reed *et al.* (1951). The first clinical use of ALA was in Germany in 1959 for the treatment of acute poisoning with *Amanita phalloides*, commonly known as 'death cap' (from mushrooms) poisoning in Europe and used in the treatment of neuropathic abnormalities (Gomes and Negrato, 2014). It is a sulphur-based antioxidant with metal chelating and antiglycation capabilities (Gomes and Negrato, 2014). ALA is a natural, potent antioxidant which is readily digested, absorbed and rapidly converted to dihydrophilic acid (DHLA) by nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) in most tissues (Kurutas, 2016). As an antioxidant, ALA can be synthesised by animals and humans. It scavenges ROS and RNS in both the fatty and watery regions of cells, in contrast to vitamin C (water soluble) and vitamin E (fat-soluble); and ALA is an anti-inflamatory agent, in both its reduced and oxidised forms (Paetau-Robinson *et al.*, 2013).

2.7.2 Pharmacokinetics of alpha-lipoic acid

Alpha-lipoic acid (ALA) is readily absorbed from an oral dose and converts easily to its reduced form, dihydrolipoic acid, in many tissues of the body. The effects of ALA and dihydrolipoic acid are present both intra- and extracellularly. ALA contains an asymmetrical carbon and, thus, has two possible optical isomers, designated as R-lipoic acid (R-ALA) and S-lipoic acid (S-ALA) (Gomes and Negrato, 2014). Naturally-occurring ALA is in the R configuration, bound to a protein; where it functions as an essential co-factor for several mitochondrial enzyme complexes, involved in energy production and the

catabolism of alpha-keto acids and amino acids (Gomes and Negrato, 2014). Nutritional supplements of ALA typically comprised either of R-ALA alone or a racemic mixture, R-ALA and S-ALA (RS-ALA). Human studies using oral racemic mixtures of ALA have observed plasma concentrations of R-ALA to be greater than that of S-ALA. One study found that, following oral administration of 50 or 600 mg racemic mixture of lipoic acid, maximum plasma concentrations (Cmax) of R-ALA doubled that of S-ALA, following 50 mg dose: 135.45 ng/mL and 67.83 ng/mL, respectively; following 600 mg dose: 1,812.32 ng/mL and 978.20 ng/mL, respectively (Breithaupt-Grogler *et al.,* 1999).

2.7.3 Mechanisms of action of alpha-lipoic acid

Alpha-lipoic acid (ALA) is a potent antioxidant in both fat- and water-soluble media. Furthermore, its antioxidant activity extends to both its oxidised and reduced forms (Gomes and Negrato, 2014). Dihydrolipoic acid is capable of neutralising free radicals and also directly regenerating vitamin C, ascorbic acid, from dehydroascorbic acid and indirectly regenerating vitamin E (Kurutas, 2016). It has been found that ALA increases intracellular glutathione and co-enzyme Q_{10} levels (Kagan *et al.*, 1990; Gomes and Negratos, 2014). Apparently, ALA is capable of chelating certain metals, including copper, manganese, and zinc (Kurutas, 2016) to form stable complexes (Paetau-Robinson *et al.*, 2013). In animal studies, ALA protects against arsenic poisoning, and, in both animal and *in vitro* studies, ALA reduces cadmium-induced hepatotoxicity. *In vitro*, ALA chelates mercury from renal ALA (Keith *et al.*, 1997).

Unlike antioxidant vitamins, ALA exhibits its antioxidants activity in both the reduced and oxidated forms. Lipoic acid is a racemic mixture. The redox couple α-lipoic/dihydrolipoic acid containing the R-enantiomer is covalently bound to a lysine residue, forming an

essential lipoamide, which functions as a co-enzyme for the E2 subunit of four multi-enzymatic mitochondrial complexes; for example, pyruvate dehydrogenase. After administration, ALA is reduced at the intracellular level by various enzymes and released into the extracellular environment as its principal metabolite, dihydrolipoic acid. As a natural compound, chemically named 1, 2-dithiolane-3-pentanoic acid ($C_8H_{14}O_2S_2$), ALA is also referred to as thioctic acid. In humans, ALA is synthesised by the liver and other tissues, and functions as a co-factor within pyruvate dehydrogenase and keto-glutarate dehydrogenase. Recently, ALA has been shown to be required for the oxidative decarboxylation of pyruvate to acetyl-CoA, the critical step bridging the gap between glycolysis and the citric acid cycle.

Since ALA is both water-and fat-soluble, it is widely distributed in plants and animals in both cellular membranes and cytosol (Gomes and Negrato, 2014). In addition, ALA and its reduced dithiol form, dihydrolipoic acid, are powerful antioxidants, and their functions described by Kurutas (2016) include: directly terminating of ROS, regeneration of exogenous and endogenous antioxidants, such as vitamins C and E, and glutathione; chelation of metal ions: and reparation of oxidised proteins. ALA is a co-factor of pyruvate dehydrogenase and α-ketolutarate dehydrogenase, an enzyme sensitive to oxidative stress; sufficient ALA supplementation stimulates activities of enzymes, thereby promoting and ameliorating oxidative phoshorylation and mitochondrial respiration. It also promotes the antioxidant defence by inducing phase two enzymes, such as glutathione synthetase to increase the concentration of the antioxidant, reduced glutathione, GSH (Kurutas, 2016).

2.7.4 Sources of alpha-lipoic acid

Alpha-lipoic acid (ALA) occurs naturally in the human diet and is found in abundance in animal tissues with high metabolic activity, such as heart, liver and kidneys, and to a lesser extent in fruits and vegetables (Shay *et al.,* 2009; Zou *et al.,* 2015). The concentrations from highest to lowest, recurring in non-animal sources are as follows: spinach, broccoli, tomato, garden pea, brussel sprouts and rice bran (Deng *et al.,* 2013). The entire ALA supplied by the diet is transported in the bloodstream to tissues and incorporated into cells; and once part of the cell, ALA must be translocated into the mitochondria, where ALA-requiring enzyme complexes exist (Gomes and Negratos, 2014). ALA of animal origin is absorbed as lipoyl-lysine, due largely because digestive proteolytic enzymes do not effectively cleave the peptide bond between the two. In mammals, ALA is not sufficiently supplied by the diet and, therefore, *de novo* synthesis takes place in the heart, liver and testes to form ALA, needed for incorporation into enzyme complexes (Morikawa *et al.,* 2001). Hence, the sources of ALA may provide very little free ALA into the circulation. It is only through supplementation that ALA reaches potentially therapeutic levels (Wollin and Jones, 2003).

2.7.5 Clinical and therapeutic roles of alpha-lipoic acid

Alpha-lipoic acid (ALA) has been reported to have regulatory components of insulin signal cascade, beneficial to skeletal muscle glucose uptake, whole body glucose tolerance, and also helps against insulin resistance in animals (Shay *et al.,* 2009). Intravenous administration of ALA is effective against diabetic polyneuropathy (Shay *et al.,* 2009) and also in the management of types 1 and 2 diabetic patients with neuropathy (Yi *et al.,* 2012; Gomes and Negratos, 2014). ALA reduces the markers of oxidative stress, nuclear factor - kappa β (NF-Kβ) activation and vascular endothelial growth in diabetic retinopathy (Lin *et*

al., 2006; Heinisch *et al.*, 2010). Topical application of ALA singly or in combination with other antioxidant agents may accelerate skin wound healings, with increased expression of vascular endothelial growth factor (VEGF) in the wound areas (Chen *et al.*, 2012). Zou *et al.* (2015) reported the anti-hypertensive effect of ALA in down-regulated CCAAT/enhancer binding protein (C/EBPβ) in cultured cardiomyocytes, exposed to hypertrophic stimuli, but this effect has not been well elucidated (Zou *et al.*, 2015).

Alpha-lipoic acid (ALA) has the ability to increase tissue glutathione (GSH) levels and present deleterious sulphydryl group modification in Ca^{2+} channels. When fed to hypertensive rats, ALA decreases systolic pressure and cystolic free Ca^{2+} and attenuates adverse renal vascular changes (Louhelainen *et al.*, 2006). It also decreases systolic pressure in high blood pressure patients and subjects with the metabolic syndrome (McMackin *et al.*, 2007). ALA regulates the expression of IL-6 (inflammatory marker), inflammatory cytokines (IL-1 and TNF-α) which occur in most inflammatory disease conditions (Ikeda and Shimada, 2001). The beneficial effects of ALA have been shown in cardiovascular diseases (Xu *et al.*, 2012), diabetic myopathy (Hegazy *et al.*, 2013), cardiac fibrosis (Lee *et al.*, 2012), ischaemic perfusion, hypertension and heart failure (Ghibu *et al.*, 2009).

There is dearth of information on the use of antioxidants, including ALA singly or in combination with imidocarb dipropionate in the amelioration of the clinical manifestations, side-effects of the drugs, oxidative stress and prevention of relapse in dogs with babesiosis. There is therefore, the need to elucidate the role of these agents as therapeutic interventions in improving and reducing economic losses caused by canine babesiosis.

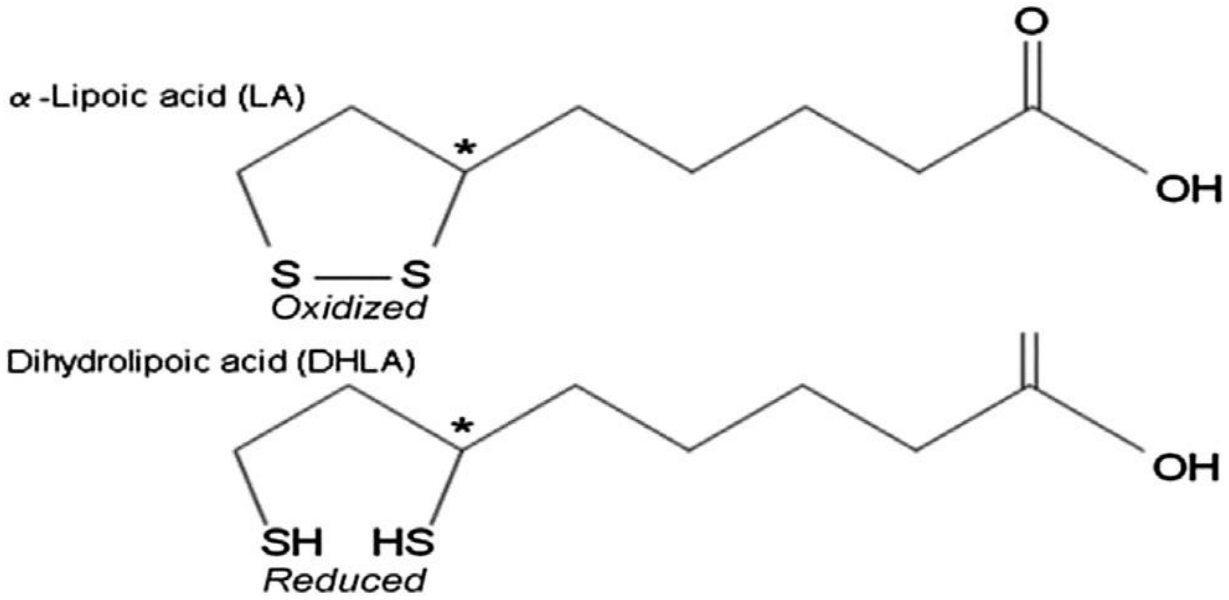


Figure 2.1: Structure of Alpha-lipoic acid

[Source: Cornille *et al.*, 2012]

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Ethical Approval

The study was approved by the Animal Use and Welfare Committee of Ahmadu Bello University, Zaria, with approval number of ABUCAUC/2016/009, and conducted in accordance with international guidelines for animal welfare.

3.2 Experimental Location

The study was conducted at the Department of Veterinary Parasitology and Entomology, Ahmadu Bello University (ABU), Zaria, located in the Northern Guinea Savannah zone of Nigeria. The main campus of ABU is located in Samaru, a suburb of Zaria in Kaduna State, Nigeria. Zaria is situated on latitude 11° 12′N and longitude 07° 37′E and at an altitude of 550-700 metres above the sea level.

3.3 Experimental Animals

Twenty-one male Nigerian indigenous dogs aged 8 - 12-month-old, weighing 8 - 12 kg, were used for the study. The dogs were obtained from Zaria and the neighbouring villages. They were pre-conditioned for a period of two weeks. During this period, blood and faecal samples were collected from all the dogs and screened for haemoparasites and helminthes, and only healthy animals were used for the study. The dogs were fed once daily and water administered *ad libitum*.

3.4 Chemical Acquisition and Preparation

3.4.1 Chemicals

Ethanol, Giemsa stain, trisodium citrate dehydrate, citric acid monohydrate, anhydrous D (+) glucose, NaCl, KCl, $NaHCO_3$, $MgCl_2.6H_2O$, $CaCl_2.6H_2O$, N(2-hydroxymethylpiprazine – N – 2- ethanesulphonic acid, sodium salt, sodium citrate, D-glucose, Na_2 ethylenediaminetetraacetic acid (EDTA), tris-HCl, NaCl were all bought from a reputable agrochemical store in Zaria, Nigeria. Alpha-lipoic acid (600 mg/tablet), General Nutrition Corporations, Pittsburgh, Philadelphia, USA, was obtained from a reputable Pharmaceutical store in Abuja, Nigeria. Imidocarb dipropionate (Forray® 65, Schering-Plough (PTY) LTD, 1600, R.S.A, G1442, Isando, South Africa), was used for the study.

3.4.2 Chemical preparation for platelet isolation from blood samples

3.4.2.1 Preparation of acid citrate dextrose, citrate glucose saline and EDTA/Tris/Saline

A total of 14 g citric acid monohydrate (39 mmol/L), 25 g trisodium citrate dehydrate (75 mmol/L) and 20 g anhydrous D (+) glucose (135 mmol/L) were all added together to make up to 1 L and buffered to pH of 7.4 to obtain the acid citrate dextrose.

A total of 294.10 g/mol of sodium citrate (10 mmol/L), 198.17 g/mol of 1% D - glucose and 58.44 g/mol saline (150 mmol/L) were added and made up to 1 L of distilled water and buffered to a pH of 6.5 to obtain citrate glucose saline.

Tris-Cl (10 mmol/L) at pH of 7.4 was added to 1 mmol/L EDTA (pH 8.0) to make Tris-buffered saline and buffered to pH of 7.4, which was the stock solution to obtain the EDTA/tris/saline. While working solution of EDTA/Tris/Saline (ETS) was prepared using

1 x citrate glucose saline (CGS) buffer (10 µL 10 x CGS + 90 µL of distilled water), which was enough for 8 acid citrate dextrose (ACD) tests.

3.5 Molecular Identification and Characterisation of *Babesia canis* spp.

3.5.1 DNA extraction

Genomic DNA was extracted from EDTA-anticoagulant whole blood samples obtained from the confirmed positive and negative donor dogs. A fresh 2 mL tube was labelled corresponding to each test sample to be processed. Lysis buffer (400 µL), proteinase K (10 µL) and whole blood sample (200 µL) were dispensed into each tube. The tubes were incubated on a heat block at 60°C for 1 h. Phenol chloroform (1:1) was added to the lysate and briefly vortexed before centrifugation at 6000 x *g* for 10 min to separate the phases. The upper layers were carefully pipetted from each tube and transferred to a new 1.5 mL tube without contamination with the white interphase. Chloroform (400 µL) was added and vortexed briefly to recover the clear layer, which was then centrifuged at 6000 x *g* for 5 min to separate the phases. The upper layers were then carefully pipetted into a new well labeled 1.5 mL tubes without contamination with the white phase. Equal volume of 100% ethanol and 20 µL of 3 M sodium acetate was added to the tubes and gently inverted severally for thorough mixing, and incubated at -20°C overnight. The mixture was centrifuged at 6000 x *g* for 10-30 min in a refrigerated centrifuge. The ethanol supernatant was removed and 70% ethanol (400 µL) was added and centrifuged at 6000 x *g* for 5 min at 4°C. This procedure was repeated thrice. The ethanol was removed and the tubes centrifuged for 30 s at 6000 x *g* to remove all traces of ethanol. The DNA was then left to air-dry for 3 - 10 min by opening the tube. The pellets were re-suspended in 50 µL of distilled water.

3.5.2 Primers and optimisation

Specific primers both forward and reverse (Table 3.1) which amplified a specific region of the 18S rRNA gene of *Babesia* (Adaszek *et al.*, 2016) were used in this study. The PCR conditions were optimised to obtain reproducible bands for the genome DNA after series of attempts. The PCR conditions used in this study were adopted after annealing condition of 60°C gave the best bands (Table 3.1).

3.5.3 Polymerase chain reaction

The DNA amplification (PCR) was carried out in a final volume of 20 µL, containing 2 µL of the template DNA, 0.2 µL of each primer (forward and reverse), 2 µL PCR buffer (including 15 mM $MgCl_2$), 2.0 µL of 2 mM dNTPs, 0.2 µL of Taq DNA polymerase and 13.40 µL Nuclease-free water (AccuPower®) to complete the total volume of the reaction. The tubes were then arranged into a programmed Applied Biosystems Veriti 96 well thermocycler (Germany). The PCR cycling conditions were initial denaturation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min, and final extension was performed at 72°C for 10 min. The PCR product was stored at 4°C for electrophoresis.

3.5.4 Electrophoresis and purification of PCR product

The amplified product was analysed by electrophoresis on 1.5% Agarose gel by aliquoting 4 µL of PCR product alongside 1 kb DNA ladder and run on gel stained with SYBR Green for 1 h at 80 volts. The PCR product was purified using MagExtraction-PCR and Gel Clean up kit (Tokyo, Japan) following the manufacture's protocol. The amplicons from the ready-made PCR was electrophoresed to monitor the size of the amplified fragments by comparing the fragments to a standard molecular weight marker (1 kb DNA ladder). A

1.5% of agarose gel was used to analyse the PCR product and it was prepared by weighing 1.5 g of agarose powder and pouring it into a conical flask containing 1 x Tris acetate-EDTA (TAE) buffer and heated in a microwave oven until is completely dissolved. This was removed from the oven and allowed to cool, and then 5 mL of staining SYBR Green (Roch Diagnostic, Mannheim, Germany) was added and mixed thoroughly.

The ends of the tray were sealed with masking tape and the comb inserted before the gel was poured, ensuring that no bubble was formed. The comb and the masking tapes were carefully removed from the gel casting trays and the tray was placed in the electrophoresis tank before 1 x TAE buffer was added to cover the gel. The molecular weight marker was loaded into the first well, the nuclease-free water was loaded into the second well as negative control and then the sample was loaded into the subsequent wells. The safety cover was put in place before power was switched on to run for 30 min at 80 V, after which the gel was removed and placed in the transilluminator (Las-4000 Chemiluscent and Fluorescence Imaging System, Fuji Film Life Sciences, USA) and documentation system for capture of the gel image. The agarose block containing the DNA template was sliced into small pieces and placed into 1.5 mL microtubes. Then, 400 µL of binding solution was added and incubated at room temperature (25 - 26°C) and vortexed every 2 - 3 min interval until the gel completely dissolved. Thereafter, 30 µL magnetic beads were added and vortexed every 10 s for 2 min and each tube placed in the magnetic stand until the magnetic beads completely separated from the specimen solution. The supernatant was carefully removed after the magnetic capture. Washing solution (600 µL) was added to the beads, and the solution was vortexed for 10 s. The tubes were placed in the magnetic stand and the beads were collected with the magnet, and the supernatant was carefully removed after the magnetic capture, after which 1 mL 75% ethanol was added to the tubes and vortexed for

seconds. This was repeated until the 75% ethanol was completely removed after a flash centrifugation. Then, 25 µL sterilised water was added and mixed well for 10 s. The solution was incubated at room temperature for 2 min, and the tubes were placed in the magnetic stand after a brief vortexing.The supernatant was collected and placed into a fresh tube.

The PCR products were analysed in a prepared 2% agarose gel electrophoresis in Tris acetate-EDTA (TAE, 0.04M Tris acetate, 1mM EDTA), stained with gel red and visualised on ultraviolent (UV) trans-illuminator (MacroVue, UV20, Hoefer, VilberLourmat, France). A 1 Kbp DNA ladder (Invitrogen, Germany) was used to comparatively determine the molecular size of the PCR amplicons. A-1µL loading dye was added to 5 µL PCR product. The solution was mixed by pipetting up and down before loading the gel. The amplicons were purified using the Toyobo® Mag-Extractor PCR and gel clean-up kit (Toyobo®, Japan) according to the manufacturer's instruction. The pure DNA was eluted in 20 µL elution buffer.

3.5.5 Sequencing and analysis of sequences

The PCR amplicons of *Babesia* spp. were selected and sequenced at the Department of Biomedical Chemistry, School of Medicine, University of Tokyo, Japan, using Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA). The reverse of the respective pairs were used for sequencing. Sequencing reaction was prepared in a 2-mL tube. All the reagents were kept on ice and used in the following order: dH_2O (0 - 0.95 µL), DNA template (0.5 - 10.0 µL), primers (2 µL), DTCS Quick start master mix (8.0 µL). The following conditions were used for the sequencing reaction: 96°C for 20 s, 50°C for 20 s (30 cycles) and 60°C for 4 min. The obtained sequences were assembled and edited

Table 3.1: Oligonucleotide sequences of primers used to identify the inoculated *Babesia canis*

Primer name	Sequence	Reference
***Babesia* genus forward**	5´-GTGAACCTTATCACTTAAAGG-3´	Duarte *et al.*, 2008
***Babesia* genus reverse**	5´-CAACTCCTCCACGCAATCG-3´	Duarte *et al.*, 2008

using BioEdit version 7.0 (Hall, 1999). Homologous sequences searches of database were performed using Basic Local Alignment Search Tool (BLAST) package (Altschul *et al.,* 1990).

3.5.6 Phylogenetic analysis

Determination of the evolutionary relationship of the *B. canis vogeli* isolates, a phylogenetic tree was generated using MEGA 7.0 software program (Kumar *et al.,* 2016). First, the 18S rRNA gene sequence data obtained from the *B. c. vogeli* isolates were separately imported into a notepad page of a computer as well as the sequences of the same organisms as the isolates from the GenBank and saved in FASTA format. The sequences of genetically distanced organisms were also retrieved from the GenBank and used as an out-group. Multiple sequence alignments were carried out using Clustal W for pair-wise comparisons and the aligned sequences were edited manually. The genetic distances between pairs of sequences were calculated using Kimura's two parameter model (Tamura *et al.,* 2013), and subsequently Neighbour-joining (NJ) algorithm was used to construct a phylogenetic tree (Saitou and Nei, 1987). A bootstrap of 1000 replicates was statistically used to evaluate the branching of the phylogenetic tree, and a boostrap value of 80% was considered significant and as an evidence of pylogenetic grouping.

3.6 Experimental Design

3.6.1 Preparation of experimental animals

The 21 Nigerian indigenous male dogs (Plate I), aged between 8 - 12 months and weighing 8 - 12 kg, were housed and acclimatised in the kennels of Ahmadu Bello University Veterinary Teaching Hospital, Zaria for two weeks before commencement of the experiment. They were assigned personal identification numbers, fed once per day on

compounded dog food, and given access to drinking water *ad libitum*. They were screened on day 0 of the experiment by measuring their weights and vital parameters (rectal temperature, respiratory and pulse rates). Blood, urine and faecal samples of each dog were collected in order to obtain the base-line values (day 0), using standard procedures, after which they were inoculated with *Babesia canis vogeli* the same day. The samples were collected at days 11 post-inoculation (PI), post-treatment (PT) 8, 15 and 43, properly labeled and analysed immediately. Treatment commenced at day 12 post-inoculation. Weekly body weight changes were taken using a weighing balance (Omron®, HN286, China).

3.6.2 Preparation of donor animal

Suspected *Babesia*-positive dogs (Plate II) were screened within Zaria and a dog that tested positive to the presence of *B. canis* was selected, splenectomised and monitored daily. Blood samples were obtained daily from the dog to determine the PCV and level of parasitaemia. Peripheral blood samples were collected during the patent parasitaemia of 10^7 (10^7 parasites per mL of blood), and the dog served as the donor animal for subsequent inoculations into the experimental dogs.

3.6.3 Experimental animal inoculation

The male dogs (n = 21) were randomly separated by an independent individual into five experimental groups: group I (n = 4), group II (n = 6), group III (n = 3), group IV (n = 5) and group V (n =3). Base-line blood parameters were evaluated. All the groups, except group I, were inoculated with 1 mL of the inoculum, containing 10^7 parasitised erythrocytes per mL from the donor animal. Parasitaemia was calculated by counting the numbers of

infected erythrocytes in at least 100 examined microscopic fields of vision (Razmi *et al.*, 2003).

3.6.3.1 Establishment of infection

Clinical signs were observed daily, including, colour of buccal and ocular membranes, behavioural changes, size of spleen and lymph nodes (Schetters, 1994). Blood samples were taken from the infected dogs every other day, until day 11, when parasitaemia and clinical manifestations of babesiosis were observed. Treatment commenced once clinical signs and parasitaemia of 10^6 were established in the experimental dogs.

3.6.4 Experimental protocol

Group I was the uninfected untreated, group II was the infected untreated, group III was the infected treated with imidocarb dipropionate (6 mg/kg subcutaneously) alone. Dogs in group IV were infected treated with ALA (15 mg/kg orally) alone, while group V was infected and treated with imidocarb dipropionate and ALA (Plate III).

3.7 Evaluation of the Effect of Treatments on Vital Parameters and Body Weight

The rectal temperature (RT), pulse and respiratory rates (PR, RR) were taken, once per day between 8.00 and 10.00 am, daily, throughout the course of the experiment, using standard procedures. Whole blood sample (1 mL) obtained from the donor dog was inoculated intravenously into each dog as described by Konto *et al.* (2014).

3.8 Evaluation of the Effect of Treatments on Haematological Parameters

3.8.1 Evaluation of the effects of treatments on blood cellular components

Blood sample (2 mL) from the cephalic vein was collected using 21 G needles and syringes into sample bottles, containing tripotassium ethylenediaminetetraacetic acid (EDTA) (2 $mg.mL^{-1}$ of blood) as anticoagulant for haematological parameters. The blood was analysed for PCV, haemoglobin (Hb) concentration, erythrocyte concentration, total and differential leucocyte counts, platelet counts, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) using the automated haematology analyser (Alfa-Swelab haematology autoanalyser, Double Medical AB, Sweden) as described by Dacie and Lewis (1991). Absolute differential leucocyte count and neutrophil-lymphocyte ratio were calculated.

3.8.2 Evaluation of the effect of treatments on glucose concentration

Blood glucose concentration was determined using glucometre strips (One Touch® Ultra Mini® Lifescan, Moulineaux, France) (Bennett *et al.*, 2017). The principle is based on blood glucose in the blood sample mixing with glucose oxidase and a mediator ferricyanide in the strip which participates in the reactions and produces ferrocene and releases electrons. The electrons make up the current that the meter then interprets as the blood glucose. The meter measured the current blood glucose level which was displayed and stored in the memory. Briefly, a drop of venous blood from the needle was transferred to the narrow channel on the test strip that has been placed on the glucometer (One Touch® Ultra Mini®) according to the manufacturers' instruction. The glucometer reading was measured and recorded in mmol/L.



Plate I: Nigerian indigenous dog



Plate II: Splenectomised *Babesia*-positive donor Nigerian indigenous – terrier cross breed dog



Plate III: Injectable imidocarb dipropionate and oral alpha-lipoic acid

3.8.3 Evaluation of the effect of treatments on erythrocyte osmotic fragility

Erythrocyte osmotic fragility was determined according to Faulkner and King (1970) and as modified by Oyewale *et al.* (1998). Briefly, sodium chloride solution (pH 7.4) was prepared at varying concentrations, 0.0%, 0.1%, 0.3%, 0.5%, 0.7%, and 0.9%. Five millilitres (5 mL) of each NaCl concentrations was placed in labeled test tubes serially in a rack. Exactly 0.02 mL of each blood sample was pipetted into each tube. The contents of the tubes were gently mixed by inverting the tubes and allowing them to stand at room temperature (24 - 25°C) for 30 min. Thereafter, the tubes were centrifuged at 1580 x *g* for 5 min using model IEC HN-SII (Damon/IEC Division, UK). The supernatant obtained from each tube was transferred to a clean glass cuvette and the absorbance of the supernatant measured spectrophotometrically with Spectronic 20 (Bausch and Lomb, USA) at a wavelength of 540 nm. The percentage of haemolysis for each sample was calculated using the following formula:

$$\text{Percentage (\%) haemolysis} = \frac{\text{Optical density of test}}{\text{Optical density of standard}} \times 100$$

3.9 Evalution of the Effect of Treatments on Serum Biochemical Parameters

Whole blood without anticoagulant was centrifuged at 1000 x *g* for 10 minutes, and the serum was separated from the blood for evaluation of biochemical parameters.

3.9.1 Evaluation of the effect of treatments on serum electrolytes

Serum electrolyte concentrations of K^+, Ca^{2+}, P and Mg were evaluated using assay kits (Spectrum Laboratories, Egyptian Company for Biotechnology, Cairo, Egypt). Sodium was determined using the colorimetric method. Briefly, the method was based on reaction of sodium with selective chromogen producing a chromophore, whose absorbance varied directly as the concentration of sodium in the serum sample rose (Henry, 1974; Tietz,

1995). Potassium concentration was determined using turbidimetric tetraphenylborate (TPB). Briefly, potassium ions and TPB form a turbid emulsion at an alkaline pH, the increase of which was measured quantitatively in a photometer at 578 nm. The increase of the absorbance (A) was directly proportional to the concentration of potassium in the serum sample (Hillman *et al.*, 1967; Tietz, 1976; Hoeflmayr, 1979). Serum chloride was determined using the colorimetric method as described by Schoenfeld and Lewellen (1964). Briefly, chloride ions and Hg II-thiocyanate formed thiocyanate ions in acidic medium. The reaction of these ions with HNO_3 and Fe III-ions gave red colour. The increasing extinction was directly proportional to the concentration of chloride ions (Passing and Bablok, 1983; Krieg *et al.*, 1986; Bablok *et al.*, 1988; Battle *et al.*, 1988; Tietz, 1995). Inorganic phosphorus was evaluated using the colorimetric method without deproteinisation (Fraser *et al.*, 1987). Briefly, this method measured inorganic phosphorus in serum accomplished by forming a phosphomolybdate complex, and in turn reducing it to a molybdenum blue colour complex (Tietz, 1990; Young, 1990).

3.9.1.1 Evaluation of the effect of treatments on magnesium

Serum samples were evaluated for the concentration of magnesium using assay kits (Fortress Diagnostics Limited, Antrim Technology Park, Antrim, BT41 1QS, United Kingdom), and calmagite method. Briefly, magnesium ions reacted with the calmagite indicator at alkaline pH to form chromophore, the rate of formation of which was measured at 520 nm (Tietz, 1995).

3.9.2 Evaluation of the effect of treatments on creatinine

Serum samples were evaluated for the activities of creatinine using assay kits (Spectrum Laboratories, Egyptian Company for Biotechnology, Cairo, Egypt) according to

colorimetric method with deproteinisation. Creatinine reacted with picric acid in alkaline solution to form a coloured complex (Tietz, 1986) known as creatinine picric acid complex as shown in equation 1:

$$\text{Creatinine} + \text{picrate} \xrightarrow{\text{NaOH}} \text{yellow} - \text{red complex} \quad (1)$$

$$\text{Concentration of creatinine (mg/dL) in the serum} = \frac{\text{A specimen}}{\text{A standard}} \times 2$$

The absorbance of the specimen and standard were measured at 546 nm using a spectrophotometer (Molecular Devices Plus Microplate®, SpectraMax Osaka, Japan).

3.9.3 Evaluation of the effect of treatments on blood urea nitrogen

Serum samples were evaluated for the concentrations of blood urea nitrogen using assay kits (Spectrum Laboratories, Egyptian Company for Biotechnology, Cairo, Egypt) according to urease-ultraviolet fixed rate (enzymatic method). Briefly, the series of reactions involved in the assay were as follows: urea was hydrolised in the presence of water and urease to produce ammonia and carbon dioxide. (Equation 2):

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2 \quad (2)$$

In the presence of glutamate dehydrogenase (GLDH) and reduced nicotinamide adenine dinucleotide (NADH), the ammonia combined with α-ketoglutarate (α-KG) to produce L-glutamate. (Equation 3):

$$H_4 + 2\alpha - KG + 2NADH \quad \rightarrow \quad 2\,L - \text{Glutamate} + 2\,NAD^+ + H_2O \quad (3)$$

The rate decreased in the NADH concentration is directly proportional to the urea concentration in the serum. It is determined by measuring the absorbance at 340 nm.

The rate decreased in the NADH concentration is directly proportional to the urea concentration in the serum. It is determined by measuring the absorbance at 340 nm.

A specimen = A1 specimen - A2 specimen

A standard = A1 standard - A2 standard

$$\text{Serum urea concentration (mg/dl)} = \frac{\text{A specimen}}{\text{A standard}} \times \text{n}$$

Where n = 107 mg/dL

3.9.4 Evaluation of the effect of treatments on bilirubin

Serum samples were evaluated for the activities of total bilirubin using assay kits (Spectrum Laboratories, Egyptian Company for Biotechnology, Cairo, Egypt) using colorimetric diazo method. Briefly, the total bilirubin concentration was determined in the presence of caffeine by the reaction with diazotized sulphanilic acid to produce an intensely coloured diazo dye (560 – 600 nm) using the colorimetric diazo method. The intensity of colour of the dye formed was proportional to the concentration of total bilirubin. (Equations 4 and 5):

$$\text{Sulphanilic acid} + NaNO_2 \xrightarrow{HCL} \text{Diazotised sulphanilic acid} \quad (4)$$

$$\text{Bilirubin} + \text{Diazotised sulphanilic acid} \xrightarrow{pH1.4} \text{Azobilirubin} \quad (5)$$

3.9.5 Evaluation of the effect of treatments on creatine kinase

Serum samples were evaluated for the activities of creatine kinase (CK) using assay kits (Spectrum Laboratories, Egyptian Company for Biotechnology, Cairo, Egypt) according to the recommendations of the International Federation of Clinical Chemistry (IFCC). Briefly, CK catalysed phosphorylation of Adenosine diphosphate (ADP), in the presence of creatine

phosphate, to form Adenosine triphosphate (ATP) and creatine. The catalytic concentration was determined from the rate of NADPH formation, measured at 340 nm, by means of hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6PDH) (Young, 2001). (Equations 6, 7, 8):

$$\text{Creatine phosphate ADP} \xrightarrow{\text{CK}} \text{Creatine} + \text{ATP} \quad (6)$$

$$\text{ATP} + \text{Glucose} \xrightarrow{\text{HK}} \text{ADP} + \text{Glucose} - 6 - \text{phosphate} \quad (7)$$

$$\text{Glucose-6-phosphate} \xrightarrow{6} - \text{Phosphogluconate} + \text{NADPH} + \text{H}^{+} \quad (8)$$

The rate decrease in the NADH concentration was directly proportional to the urea concentration in the specimen, and was determined by measuring the absorbance at 340 nm.

3.9.6 Evaluation of the effect of treatments on serum proteins

3.9.6.1 Evaluation of the effect of treatments on total protein

Serum samples were evaluated for the concentrations of total protein using assay kits (Fortress Diagnostics Limited, Antrim Technology Park, Antrim, BT41 1QS, United Kingdom), according to Biuret method (Henry, 1974). Copper ions reacted in alkaline solution, with protein peptide bonds to give a purple coloured Biuret complex. The amount of complex formed was directly proportional to the amount of protein in the serum sample (Tietz, 1995).

3.9.6.2 Evaluation of the effect of treatments on albumin

Serum samples were evaluated for the activities of albumin (Spectrum Laboratories, Egyptian Company for Biotechnology, Cairo, Egypt) using modified bromocresol green

colorimetric method (Doumas *et al.*, 1971). Briefly, albumin was measured based on its binding to the indicator dye bromocresol green (BCG) in pH 4.1 to form a blue-green coloured complex. The intensity of the blue-green colour was directly proportional to the concentration of albumin in the serum sample. It was determined by monitoring the increase in absorbance at 623 nm, or 578 nm (Grant *et al.*, 1987; Tietz, 1990). (Equation 9):

$$\text{Albumin} + \text{BCG} \xrightarrow{\text{pH4.1}} \text{Albumin} - \text{BCG Complex} \quad (9)$$

3.9.7 Evaluation of the effect of treatments on serum trace elements

3.9.7.1 Evaluation of the effect of treatments on copper

Serum samples were evaluated for the concentration of copper using assay kits (Spectrum Laboratories, Egyptian Company for Biotechnology, Cairo, Egypt) using colorimetric method with Dibrom-PAESA. Briefly, the colorimetric method with Dibrom-PAESA was used in which copper forms with 4-(3, 5-dibromo-2-pyridylazo)-N-ethyl-sulfopropylaniline a chelate complex. The increase of absorbance of this complex was measured and was proportional to the concentration of total copper in the serum (Abe *et al.*, 1989).

3.9.7.2 Evaluation of the effect of treatments on zinc

Serum samples were evaluated for the concentration of zinc using assay kits (Spectrum Laboratories, Egyptian Company for Biotechnology, Cairo, Egypt) and colorimetric method with 5-Brom-PAPS. Briefly, zinc formed a red chelate complex with 2-(5-Brom-2-pyridylazo)-5-(N-propyl-N-sulphopropylamino)-phenol. The increase of absorbance was measured and proportional to the concentration of total zinc in the serum (Johnsen and Eliasson, 1987).

3.9.7.3 Evaluation of the effect of treatments on iron

Serum samples were evaluated for the concentration of iron using assay kits (Fortress Diagnostics Limited, Antrim Technology Park, Antrim, BT41 1QS, United Kingdom), using ferrene colorimetric method. Briefly, iron was dissociated from the transferring-iron in weakly acidic medium. Liberated iron was reduced into bivalent form by means of ascorbic acid. Ferrous ions with Nitro-PAPS (2-(5-nitro-2-pyridyl-azo)-5-(n-propyl-n-(3-sulfopropyl-amino-phenol) gave coloured complex. (Equations 10, 11):

$$\text{Transferrin}(Fe^{3+})_2 \rightarrow 2Fe^{2+} + \text{Transferin} \quad (10)$$

$$Fe^{2+} \xrightarrow{\text{Nitro-PAPS}} \text{Coloured complex} \quad (11)$$

The intensity of the coloured complex formed was proportional to the iron concentration in the serum (Tietz, 1995).

3.9.8 Evaluation of the effect of treatments on liver enzyme activities

3.9.8.1 Evaluation of the effect of treatments on alanine aminotransferase

Serum samples were evaluated for the activities of alanine aminotransferase (ALT) using assay kits (Fortress Diagnostics Limited, Antrim Technology Park, Antrim, BT41 1QS, United Kingdom). The principle was based on colorimetric with standard to devaluate the quantitative ALT in the serum. (Equation 12):

$$\alpha - \text{oxoglutarate} + \text{L} - \text{aspartate} \xrightarrow{\text{GPT}} \text{L} - \text{glutamate} + \text{Pyruvate} \quad (12)$$

3.9.8.2 Evaluation of the effect of treatments on aspartate transaminase

Serum samples were evaluated for the activities of aspartate transaminase (AST) using assay kits (Fortress Diagnostics Limited, Antrim Technology Park, Antrim, BT41 1QS,

United Kingdom). The principle was based on colorimetric method to evaluate the quantitative AST in the serum. (Equation 13):

$$\alpha - \text{oxoglutarate} + \text{L} - \text{aspartate} \xrightarrow{\text{GOT}} \text{L} - \text{glutamate} + \text{Oxaloacetate} \quad (13)$$

The absorbance of the increasing amounts of pyruvate corresponds to the transaminase activities in units per litre.

3.9.8.3 Evaluation of the effect of treatments on alkaline phosphatase

Serum samples were evaluated for the activities of alkaline phosphatase (ALP) using assay kits (Spectrum Laboratories, Egyptian Company for Biotechnology, Cairo, Egypt). The principle was based on kinetic method according to the International Federation of Clinical Chemistry (IFCC). Briefly, p-Nitrophenyl phosphate is converted to p-Nitrophenol and phosphate by alkaline phosphate. The increase of absorption at 405 nm was proportional to the alkaline phosphatase concentration in the serum sample (Moss, 1982; Tietz *et al.,* 1983; Moss *et al.,* 1987; Zawta *et al.,* 1994).

3.9.8.4 Evaluation of the effect of treatments on gamma-glutamyl transferase

Serum samples were evaluated for the activities of gamma-glutamyl transferase (γ-GT) using assay kits (Spectrum Laboratories, Egyptian Company for Biotechnology, Cairo, Egypt). The principle is based on kinetic colorimetric method according to Szasz *et al.* (1974). The rate of liberation of yellow coloured indicator 5-amino-2-nitrobenzoate was directly proportional to γ-GT activity in the serum sample and quantitated by measuring the increase in absorption at 405 nm (Szasz *et al.,* 1974; Persijn and Van der Slike, 1976; Heersink *et al.,* 1980; Saw *et al.,* 1983). (Equation 14):

$$L-\gamma-Glutamyl-3-carboxy-4-nitroanilide + L-\gamma-Glutamylglycine + 5-amino-2-nitrobenzoate \quad (14)$$

3.9.9 Evaluation of the effect of treatments on MDA concentration and activities of antioxidant enzymes

3.9.9.1 Evaluation of the effect of treatments on erythrocyte malondialdehyde concentration

Blood sample (2 mL) obtained in EDTA from each experimental dog was centrifuged at 3000 x *g* and the plasma discarded. The erythrocytes were washed thrice in cold isotonic saline (0.9%, w/v). Erythrocyte packets were prepared and used to assay for erythrocyte MDA concentrations using the double-heating method of Draper and Hadley (1990), as modified by Ambali *et al.* (2011). Erythrocyte MDA concentration was assayed using commercial kits (Oxford Biomedical Research, Michigan, USA). The principle of the method was based on spectrophotometric measurement of the colour produced during the reaction to 2-thiobarbituric acid (TBA) with MDA at 25°C.One molecule of MDA reacted with two molecules of 2-thiobarbituric acid *via* a Knoevenagel-type condensation to yield a chromophore with absorbance maximum at 532 nm.

3.9.9.2 Procedure for isolation of platelets from blood samples andevaluation of the effect of treatments on platelet glutathione peroxidase

Blood samples (3 mL) from the dogs were added to 0.5 mL acid citrate dextrose (ACD) and centrifuged at 140 x *g* for 20 min at room temperature to obtain the supernatant platelet rich plasma (PRP). The PRP was pippeted into new labeled tubes. Equal volume of PRP and citrate glucose saline (pH 6.5) buffer was added to the tubes containing the PRP. The mixture was centrifuged at 400 x *g* for 10 min at room temperature to obtain the platelet pellet after discarding the plasma poor platelet (PPP) supernatant. The platelet pellet was gently washed thrice using 500 µL citrate glucose saline buffers. The platelet pellet was

resuspended in ETS buffer (pH 7.4) and kept at 4°C until use. Platelets were prepared and lysed according to the method of Shalayel *et al.* (2014).

Thrombocytes were evaluated for the antioxidant activities of glutathione peroxidase (GPx) using assay kits (Fortress Diagnostics Limited, Antrim Technology Park, Antrim, BT41 1QS, United Kingdom). Glutathione peroxidase catalised the oxidation of glutathione (GSH) by cumene hydroperoxide. The oxidised glutathione was converted to the reduced form in the presence of glutathione reductase and NADPH. The NADPH was oxidised to $NADP^+$ simultaneously. The decrease in absorbance at 340 nm was measured (Thomas *et al.*, 1990). (Equations 15 and 16):

$$ROOH + 2GSH \xrightarrow{GPx} ROH + GSSG + H_2O \quad (15)$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} NADP^+ + 2GSH \quad (16)$$

3.9.9.3 Evaluation of the effect of treatments on superoxide dismutase

Serum samples were evaluated for the antioxidant activities of superoxide dismutase (SOD) using assay kits (Sigma-Aldrich, St. Louis, Missouri, USA), according to colorimetric method. Briefly, SOD assay utilised Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt) that produced a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with O_2 was linearly related to the xanthine oxidase (XO) activity, and it was inhibited by SOD. The absorbance at 440 nm was proportional to the amount of superoxide anion, the SOD activity as an inhibition activity was quantified by measuring the decrease in colour development at 440 nm (Gu and Chang, 2009).

3.9.10 Evaluation of the effect of treatments on cytokine concentration

3.9.10.1 Evaluation of the effect of treatments on serum tumour necrosis factor-alpha

Serum TNF-α concentrations were assayed using commercial kits (Elabscience Biotechnology Co., Ltd., Wuhan, 430070 Hubei, China), and the Sandwich-ELISA method. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to canine TNF-α. Standards or serum samples were added to the appropriate micro-ELISA plate wells and combined with the specific antibody. Thereafter, a biotinylated detection antibody specific for TNF-α and Avidin-horseradish peroxidase (A-HRP) conjugate were added to each micro-plate well successively and incubated. Free components were washed away. The substrate solution was added to each well. Only those wells that contained TNF-α, biotinylated detection antibody and A-HRP conjugate appeared blue in colour. The enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution and the colour turned yellow. The optical density was measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The optical density value was proportional to the concentration of TNF-α. The concentration of TNF-α in the samples was calculated by comparing the optical density of the samples to the standard curve.

3.10 Data Analyses

The percentage of nucleotide variation among sequences of the species was determined by pair-wise comparison, using maximum composite likelihood model in MEGA 7.0 software (Kumar *et al.,* 2016). Data obtained were expressed as mean ± SEM. Values obtained for the parameters were subjected to one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison *post-hoc* test, to determine the level of significance between the different durations within each group. Two-way ANOVA, followed by Bonferroni *post-hoc* test was used to evaluate the level of significance between the groups. GraphPad Prism

version 5.0 for windows (Graphpad Software, San Diego, California, USA) was used for the analyses. Values of $P < 0.05$ were considered to be significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Molecular Identification and Characterisation of *Babesia canis* spp.

The results (Plates IV and V) showed 576 bp gene sequence gel electrophorogram (Plate IV) of *B. canis vogeli* (Appendices I and II) isolate from Zaria, which has 100% identity (Plate V) with *B. canis vogeli* isolates from India, Brazil and a cat in Taiwan.

4.2 Effect of Treatments on Clinical Signs

Most of the clinical manifestations (Table 4.1) were observed on day 12 post-inoculation (PI) (day 0 PT). Fever was observed in individual dogs at different days, but was recorded as early as day 2 PI in infected untreated group (50%); and days 3 (60%) and 5 (40%) PI in infected + ALA group. Ocular discharge (Plate VI) was observed in infected + imidocarb group (66.7%) from days 6 - 21 PIES (9 PT). Pale mucous membrane (Plate VII) was recorded from days 6 - 29 PI (17 PT) in infected + ALA group (100%), while lymphadenopathy was observed from days 2 - 19 PI (7 PT), throughout the course of the infection in infected + imidocarb + ALA (100%) and from day 3 PI till the end of the experiment in infected untreated (50%) group. Generalised lymphadenopathy (100%) was observed in all the groups, except in infected + ALA (80%) group, while lethargy (100%) (Plate VIII) and myalgia (33.3%) were recorded in infected untreated group. There was no dryness of muzzle, ocular discharge and myalgia in the infected + imidocarb + ALA dogs. Sunken eyeballs were observed from day 14 PI (2 PT) till the end of the experiment in all the groups, with infected + imidocarb + ALA group (66.7%) having the highest prevalence. Splenomegaly was observed in all the infected dogs at day 11 PI (100%). Two weeks after the treatment, percentage splenomegaly varied among the groups: the infected untreated (100%), infected + imidocarb (33%), infected + ALA (20%), and infected + imidocarb +

ALA (0%). Co-administration of imidocarb dipropionate and ALA ameliorated the splenomegaly often associated canine babesiosis.

4.3 Effect of Treatments on Vital Parameters

4.3.1 Effect of treatments on rectal temperature

Generally, all the rectal temperatures obtained in the study were within the normal range of 37.6°C to 39.5°C. The uninfected untreated dogs (38.18 ± 0.09°C) had a lower ($P < 0.01$) RT than the infected + ALA dogs (38.90 ± 0.11°C) and infected + imidocarb + ALA dogs (38.39 ± 0.06°C) at day 8 PT (Figure 4.1). Similarly, infected + ALA dogs (38.90 ± 0.11°C) had a higher ($P < 0.001$) RT than that of dogs in the infected untreated (38.06 ± 0.16°C) and infected + imidocarb (38.13 ± 0.08°C; $P < 0.01$) groups, respectively. At day 15 PT, the RT in uninfected untreated dogs (38.13 ± 0.08°C) was significantly ($P < 0.01$) lower than the values obtained in the infected untreated (38.93 ± 0.18°C) and infected + ALA (38.83 ± 0.23°C) groups. The infected + imidocarb dogs had lower ($P < 0.001$) RT (37.92 ± 0.19°C) compared to the infected untreated (38.93 ± 0.18°C) and infected + ALA (38.83 ± 0.23°C) dogs. The RT recorded was lower ($P < 0.001$) than those obtained in the infected + imidocarb + ALA dogs (37.87 ± 0.16°C) compared to the uninfected untreated (38.13 ± 0.08°C), infected untreated (38.93 ± 0.18°C) and infected + ALA (38.83 ± 0.23°C) dogs. At day 43 PT, dogs in the uninfected untreated group (37.69 ± 0.12°C) had higher ($P < 0.05$) RT than those in the infected + imidocarb + ALA group (37.61 ± 0.13°C). Similarly, infected + ALA dogs (38.13 ± 0.11°C) had higher ($P < 0.001$) RT, compared to infected + imidocarb dogs (37.51 ± 0.20°C). Comparison within each group showed that the RT at base-line (day 0) (38.84 ± 0.29°C) and day 11 PI (38.74 ± 0.07°C) in the infected + imidocarb dogs, was significantly ($P < 0.05$) higher than that obtained at days 15 PT (37.92 ± 0.19°C) and 43 PT (37.51 ± 0.20°C; $P < 0.01$).

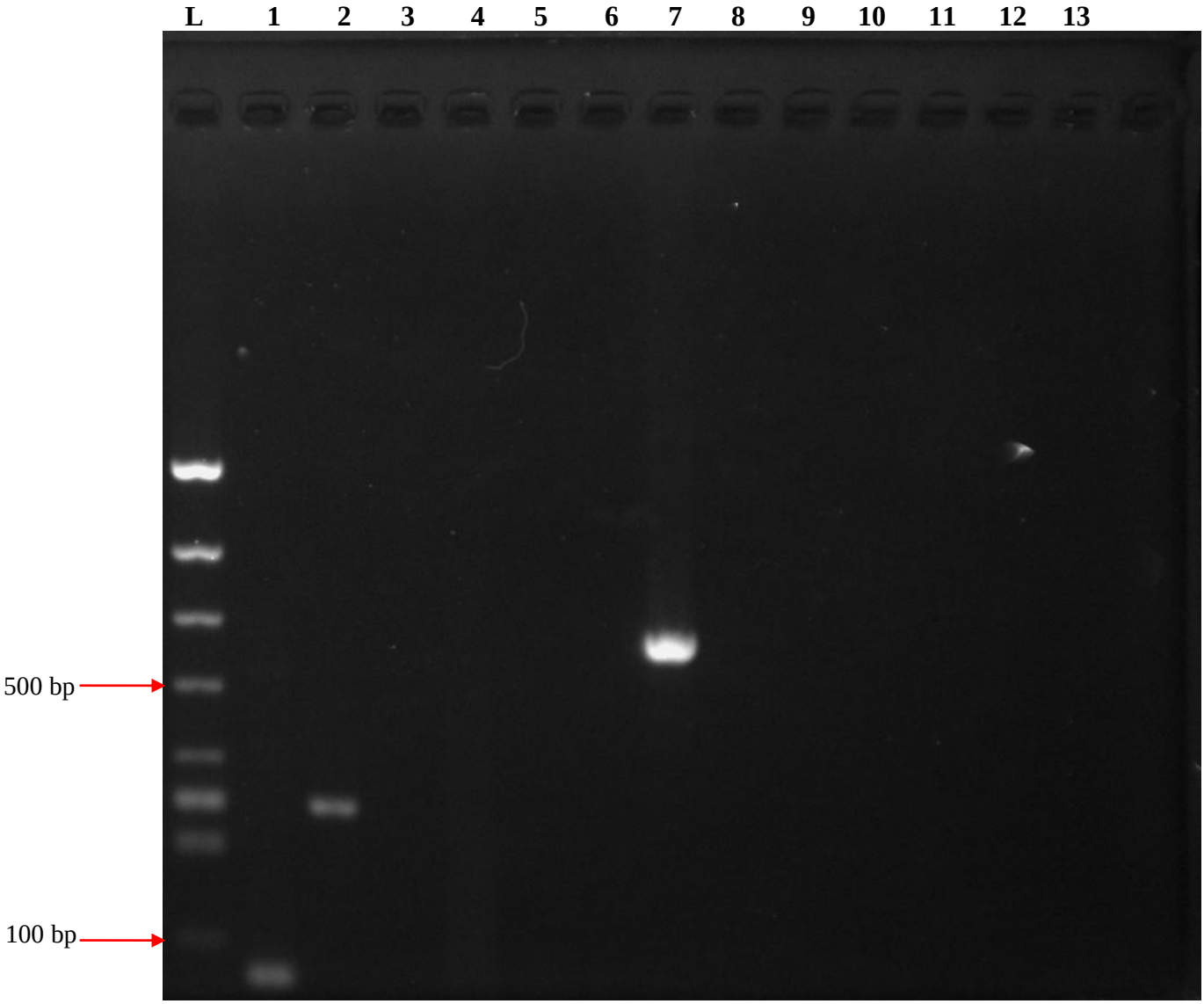


Plate IV: Agarose gel electrophoresis of amplified gene products of *Babesia canis vogeli* isolates in Zaria using specific primers separated on 1.5% agarose gel stained with SYBR Green

Lanes: {L: Marker, 1: *Babesia canis canis* (pilot study), 2: *Babesia canis rossi* (pilot study), 3-6: Apicomplexa, *Babesia gibsoni*, 7: *Babesia canis vogeli* (present study), 8-9 (Negative control)

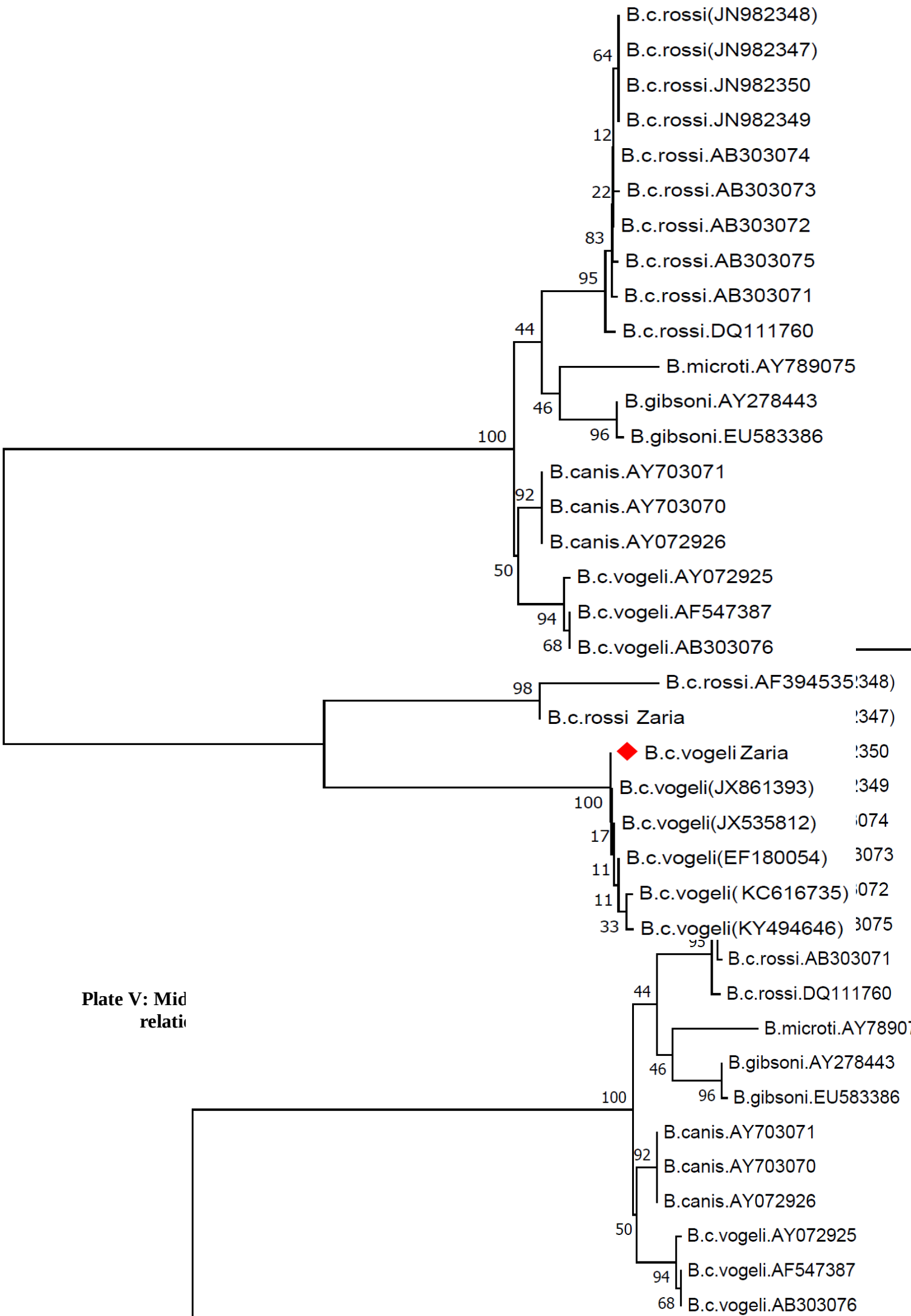


Plate V: Mid
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Table 4.1: Clinical manifestations observed in dogs experimentally infected with *Babesia canis vogeli*

Clinical manifestations	Infected untreated	Infected + Imidocarb	Infected + ALA	Infected + Imidocarb + ALA
Loss of appetite	D 0 PT (16.7%)	D 0 PT (33.3%)	D 0 PT - 1 PT (100%)	D 1 PT (33.3%)
Fever	D 2 - 9 PI (50%)	D 6 PI (33.3%)	D 3 PI (60%), D 3 PT (40%)	D 3 PI (33.3%)
Dullness	D 0 PT (33.3%)	D 0 PT (16.7%)	D 0 PT (60%)	D 0 PT (33.3%)
Dry muzzle	D 0 PT (100%)	D 0 PT (33.3%)	D 0 PT (40%)	None (0%)
Ocular discharge	D 6 PT - 9 PT (50%)	D 6 PI - 9 PT (66.7%)	D 0 PT - 9 PT (60%)	None (0%)
Pale mucous membrane	D 0 PT - 7 PT (83.3%)	D 0 PT - 7 PT (100%)	D 6 PI - 17 PT (100%)	D 0 PT - 8 PT (100%)
Lymphnode enlargement	D 3 PI - end (50%)	D 6 PI - 8 PT (100%)	D 6 PI - 8 PT (60%)	D 2 PI - 7 PT (100%)
Generalised lymphadenopathy	D 0 PT - end (100%)	D PT 0 - 10 PT (100%)	D 0 PT - 17 PT (80%)	D 8 PI - 9 PT (100%)
Sunken eyeballs	D 2 PT - end (50%)	D 8 PT (33.3%)	D 2 PT - end (40%)	D 3 PT - 9 PT (66.7%)
Lethargy	D 0 PT - end (100%)	D 0 PT (33.3%)	None (0%)	D 0 PT (33.3%)
Myalgia	D 0 PT - end (33.3%)	None (0%)	None (0%)	None (0%)
Splenomegaly	D 11 PI - end (100%)	D 11 PI - 3 PT (33.3%)	D 11 PI (100%), PT (20%)	D 11 PI (100%), PT (0%)
Number of dogs	6	3	5	3

D = Day of onset of clinical manifestations, PI = Post-inoculation, PT = Post-treatment, ALA = Alpha-lipoic acid
Values in perenthesis are percentages of dogs involved



Plate VI: Ocular discharge in Nigerian indigenous dogs infected with *Babesia canis vogeli*



Plate VII: Pale mucous membrane in Nigerian indigenous dogs infected with *Babesia canis vogeli*



Plate VIII: Lethargy in Nigerian indigenous dog infected with *Babesia canis vogeli*



Plate IX: Body weight loss in Nigerian indigenous dog infected with *Babesia canis vogeli*

Dogs in the infected + ALA group had higher ($P < 0.05$) RT recorded, at PT days 8 (38.90 ± 0.11°C) and 15 (38.83 ± 0.23°C), than that at day 43 PT (38.13 ± 0.11°C). Dogs in the infected + imidocarb + ALA group had a higher ($P < 0.05$) RT at base-line (38.50 ± 0.33°C) than that at day 43 PT (37.61 ± 0.13°C).

4.3.2 Effect of treatments on pulse rate

There was no significant ($P > 0.05$) difference in the pulse rate (PR) within the groups (Figure 4.2). There was no significant ($P > 0.05$) difference in the pulse rate between the groups at base-line and day 11 PI. At day 8 PT, uninfected untreated dogs (84.50 ± 2.54 beats/min), infected + imidocarb (82.50 ± 0.00 beats/min) and infected + imidocarb + ALA (74.83 ± 5.93; $P < 0.001$) had a lower ($P < 0.01$) PR than the infected + ALA dogs (122.05 ± 16.15 beats/min). Infected untreated dogs (107.83 ± 9.49 beats/min) had a higher ($P < 0.05$) PR, compared to the infected + imidocarb +ALA dogs (74.83 ± 5.93 beats/min). At day 15 PT, uninfected untreated dogs (83.75 ± 1.24 beats/min), infected + imidocarb (124.72 ± 13.31 beats/min; $P < 0.001$) and infected + imidocarb + ALA had lower ($P < 0.05$) PR compared to dogs in the infected + ALA group (122.05 ± 16.15 beats/min). Lower ($P < 0.05$) PR was recorded in the infected + imidocarb + ALA dogs (77.33 ± 7.16 beats/min) compared to the infected untreated (109.07 ± 5.80 beats/min). There was no significant ($P > 0.05$) difference in PR values between the groups at day 43 PT (Figure 4.2).

4.3.3 Effect of treatments on respiratory rate

The infected untreated dogs had higher ($P < 0.01$) respiratory rate (RR) (Figure 4.3) at base-line (29.73 ± 1.54 cycles/min) compared to PT days 8 (22.25 ± 0.35 cycles/min) and 43 (21.70 ± 0.53 cycles/min).

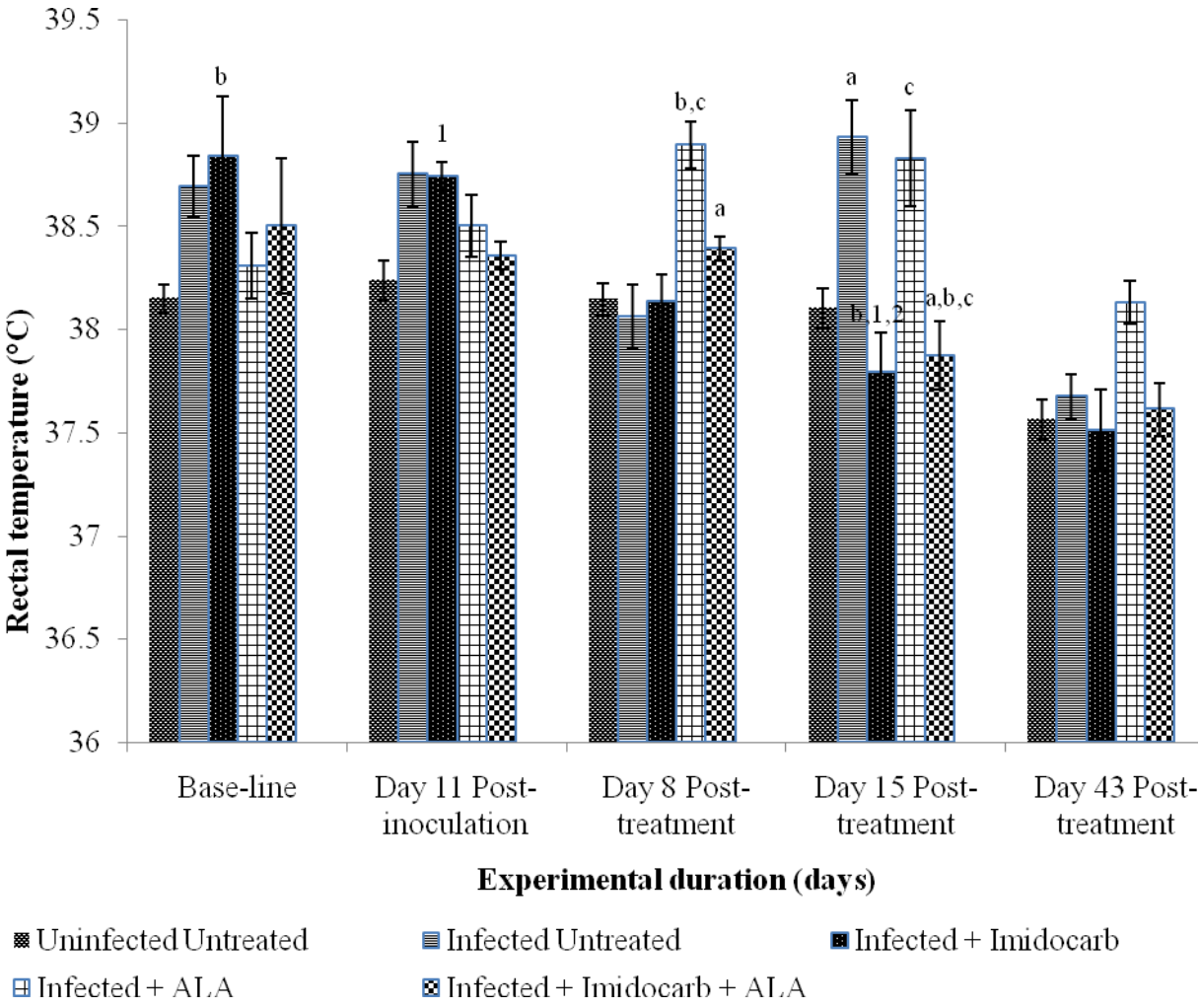


Figure 4.1: Effect of treatments on rectal temperature of dogs experimentally infected with *Babesia canis vogeli*

[1,2,3,4]P< 0.05 versus base-line, days 11 PI, 8 PT and 15 PT
[a,b,c]P < 0.05 versus uninfected untreated, infected untreated and infected + imidocarb groups, respectively

Normal range: 37.6°C to 39.5°C

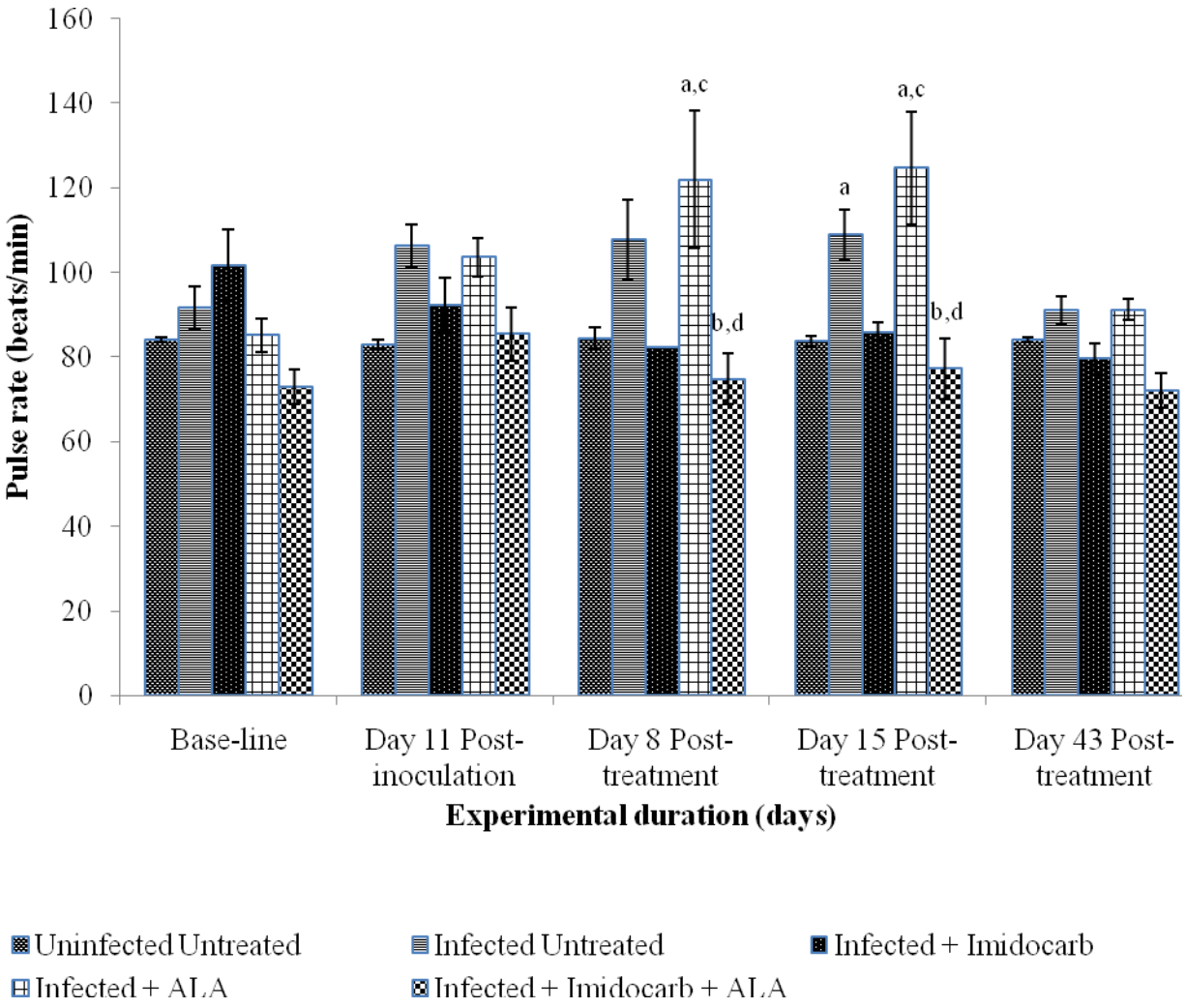


Figure 4.2: Effect of treatments on pulse rate of dogs experimentally infected with *Babesia canis vogeli*

[a,b,c,d]P < 0.05 versus uninfected untreated, infected untreated, infected + imidocarb and infected + imidocarb + ALA groups, respectively

Normal range: 90 - 120 beats/min

Respiratory rate at day 15 PT (27.83 ± 0.96 cycles/min) was higher ($P < 0.05$) than that recorded at day 43 PT (21.70 ± 0.53 cycles/min) in the infected untreated group. There was no significant ($P > 0.05$) difference within the dogs in the infected + imidocarb. In the infected + ALA dogs, base-line RR (28.24 ± 0.76 cycles/min) was higher ($P < 0.01$) than at day 43 PT (20.96 ± 1.16 cycles/min). In the infected + imidocarb +ALA dogs, base-line RR (27.33 ± 1.04 cycles/min) was higher than the values recorded at days 11 PI (21.67 ± 0.34 cycles/min; $P < 0.05$) and 43 PT (19.93 ± 0.70 cycles/min; $P < 0.01$). There was no significant ($P > 0.05$) difference between the groups at base-line, days 11 PI, 8 PT, 15 PT and 43 PT, respectively.

4.4 Effect of Treatments on Body Weight

The infected untreated group had a lower ($P < 0.05$) body weight (Figure 4.4) at base-line (10.00 ± 0.63 kg) compared to day 11 PI (10.17 ± 0.70 kg), but higher ($P < 0.05$) at day 11 PI compared to the value obtained at PT (7.50 ± 0.50 kg). There was no significant ($P > 0.05$) difference in body weight within the infected + imidocarb dogs. In the infected + ALA dogs, body weight at base-line (9.00 ± 0.63 kg) was lower ($P < 0.05$) than at day 11 PI (9.80 ± 0.58 kg; $P < 0.01$), and higher than at PT (7.00 ± 0.32 kg). In the infected + imidocarb + ALA dogs, higher ($P < 0.01$) body weight was recorded at day 11 PI (12.00 ± 0.00 kg), compared to the values at base-line (10.33 ± 0.33 kg) and PT (9.00 ± 0.00 kg).

There was significantly ($P < 0.001$) higher body weight in the uninfected untreated dogs (12.25 ± 0.48 kg) compared to the infected untreated (7.50 ± 0.50 kg); infected + imidocarb (9.00 ± 1.00 kg; $P < 0.05$), infected + ALA (7.00 ± 0.32 kg; $P < 0.05$) and infected + imidocarb + ALA (9.00 ± 0.00 kg; $P < 0.05$), respectively. Body weight decreased in all the infected groups at post-treatment irrespective of the therapies that were instituted.

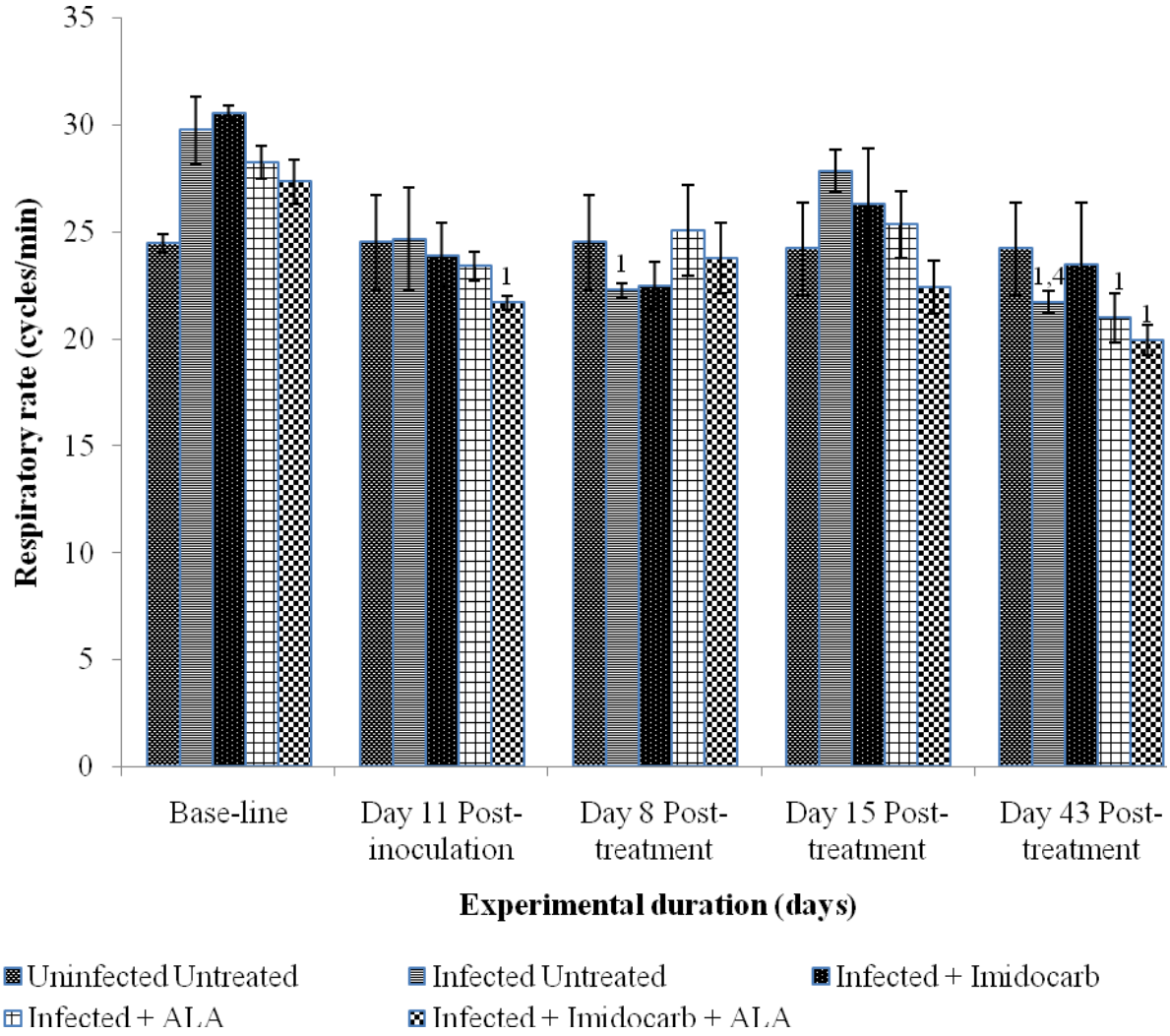


Figure 4.3: Effect of treatments on respiratory rate of dogs experimentally infected with *Babesia canis vogeli*

[1,4]P < 0.05 versus base-line and day 15 PT, respectively

Normal range: 18 - 34 cycles/min

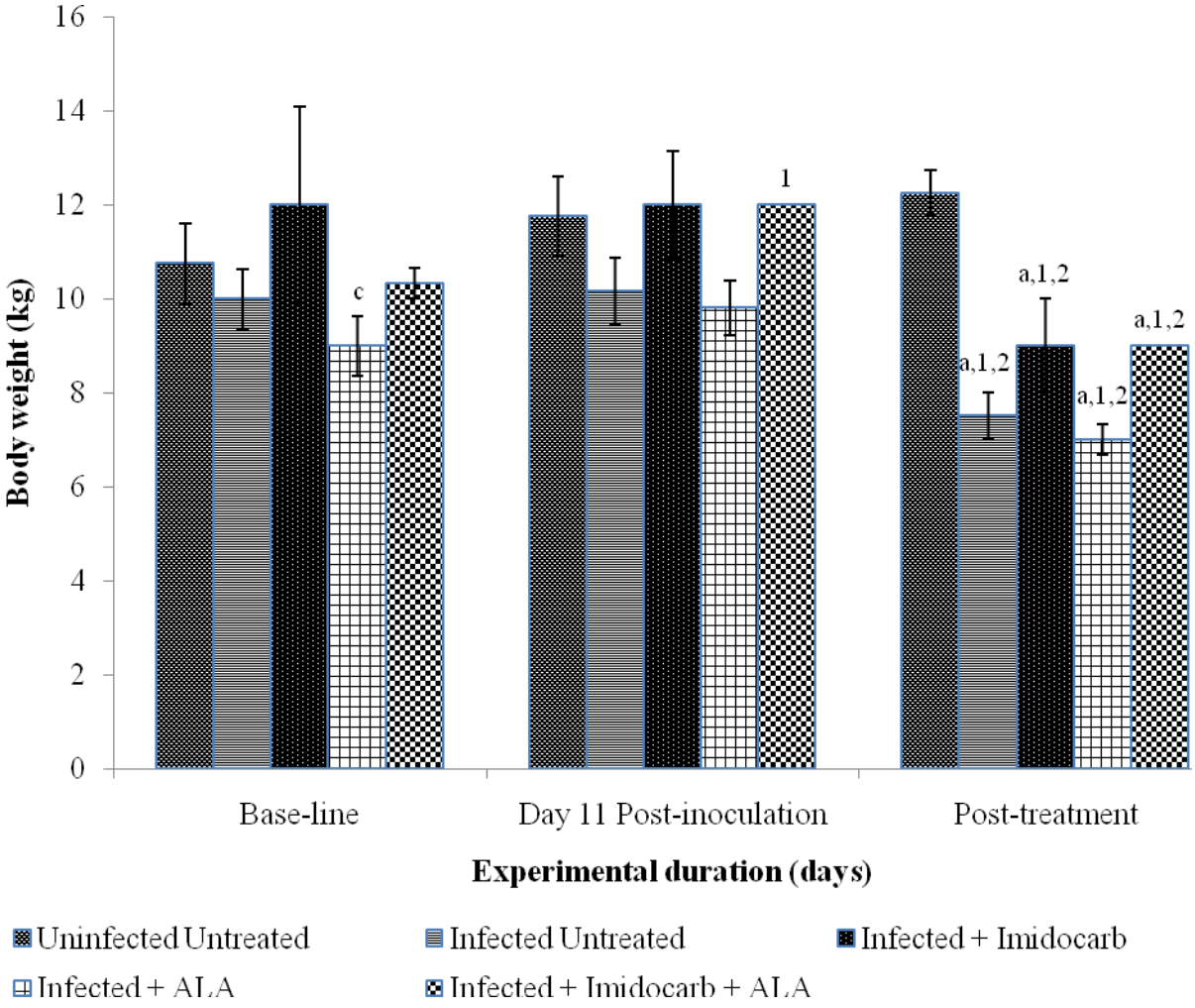


Figure 4.4: Effect of treatments on body weight of dogs experimentally infected with *Babesia canis vogeli*

[1,2]P < 0.05 versus base-line and day 11 PI, respectively
[a,c]P < 0.05 versus uninfected untreated and infected + imidocarb groups, respectively

4.5 Effect of Treatments on Blood Cellular Components

4.5.1 Effect of treatments on packed cell volume

There was no significant ($P > 0.05$) change in the PCV within the groups (Figure 4.5) at base-line, day 11 PI, PT days 8, 15 and 43. At base-line, there was no significant ($P > 0.05$) difference in the PCV between the groups. At day 11 PI, uninfected untreated dogs (50.03 ± 2.80%) had a higher ($P < 0.01$) PCV compared to that of the infected untreated (33.12 ± 3.33%), infected + imidocarb (32.20 ± 1.01%); infected + ALA (31.98 ± 3.04%) and infected + imidocarb + ALA (33.00 ± 4.05%) dogs, respectively. Insignificant ($P > 0.05$) difference was recorded at day 8 PT in the PCV between all groups. At day 15 PT, uninfected untreated dogs (48.50 ± 3.13%) had a higher ($P < 0.01$) PCV than infected untreated dogs (32.08 ± 1.40%); infected + imidocarb (29.70 ± 1.99%; $P < 0.01$); infected + ALA (34.64 ± 4.25%; $P < 0.05$); but no significant ($P > 0.05$) difference with the PCV in the infected + imidocarb + ALA (41.13 ± 2.96%) dogs. There was no significant ($P > 0.05$) change in the PCV between the groups at day 43 PT. Decreased PCV were recorded in all the infected dogs at day 11 PI and day 15 PT except in the infected + imidocarb + ALA dogs that had normal PCV at day 15 PT.

4.5.2 Effect of treatments on red blood cell count

Difference in RBC count (Figure 4.6) within the groups was insignificant ($P > 0.05$) at base-line, days 11 PI, 8 PT, 15 PT and 43 PT, respectively. At day 11 PI, the RBC count in the uninfected untreated dogs (6.71 ± 0.38% x 10^{12}/L) was significantly ($P < 0.01$) higher than the infected untreated dogs (4.64 ± 0.51 x 10^{12}/L); infected + ALA dogs (4.68 ± 0.51 x 10^{12}/L; $P < 0.05$); and infected + imidocarb + ALA dogs (4.67 ± 0.63 x 10^{12}/L; $P < 0.05$); but no significant ($P > 0.05$) difference with infected + imidocarb dogs (5.07 ± 0.44 x 10^{12}/L). At day 8 PT, there was no significant ($P > 0.05$) change in the RBC count between

the groups. At day 15 PT, the RBC count was higher ($P < 0.05$) in the uninfected untreated dogs ($6.57 \pm 0.40 \times 10^{12}$/L), compared to the infected untreated dogs ($4.68 \pm 0.21 \times 10^{12}$/L); infected + imidocarb dogs ($4.36 \pm 0.34 \times 10^{12}$/L); but no significant ($P > 0.05$) difference compared to infected + ALA dogs ($5.28 \pm 0.63 \times 10^{12}$/L) and infected + imidocarb + ALA dogs ($6.10 \pm 0.44 \times 10^{12}$/L), respectively. No significant ($P > 0.05$) difference in the RBC count between the groups was recorded at day 43 PT.

4.5.3 Effect of treatments on haemoglobin concentration

The Hb concentration in the uninfected untreated dogs (15.18 ± 1.24 g/dL) at day 11 PI, was higher ($P < 0.05$) than that obtained in the infected untreated dogs (10.90 ± 1.07 g/dL); and infected + ALA dogs (10.52 ± 0.91 g/dL), respectively. There was no significant ($P > 0.05$) difference in the Hb concentration in the uninfected untreated (15.18 ± 1.24 g/dL) dogs and infected + imidocarb dogs (11.77 ± 1.02 g/dL); infected + imidocarb + ALA dogs (10.87 ± 1.14 g/dL), respectively. At day 8 PT, the Hb concentration between the groups did not differ.

At day 15 PT, the uninfected untreated dogs (14.73 ± 1.26 g/dL) had a higher ($P < 0.05$) Hb concentration, compared to the infected untreated (10.80 ± 0.49 g/dL); and infected + imidocarb (10.31 ± 0.56 g/dL), respectively. There was no significant ($P > 0.05$) difference in the Hb concentration between the infected untreated dogs (10.80 ± 0.49 g/dL) and infected + imidocarb + ALA dogs (13.80 ± 0.91 g/dL), respectively.At day 43 PT, the Hb concentrations between the groups were not significantly different (Figure 4.7).

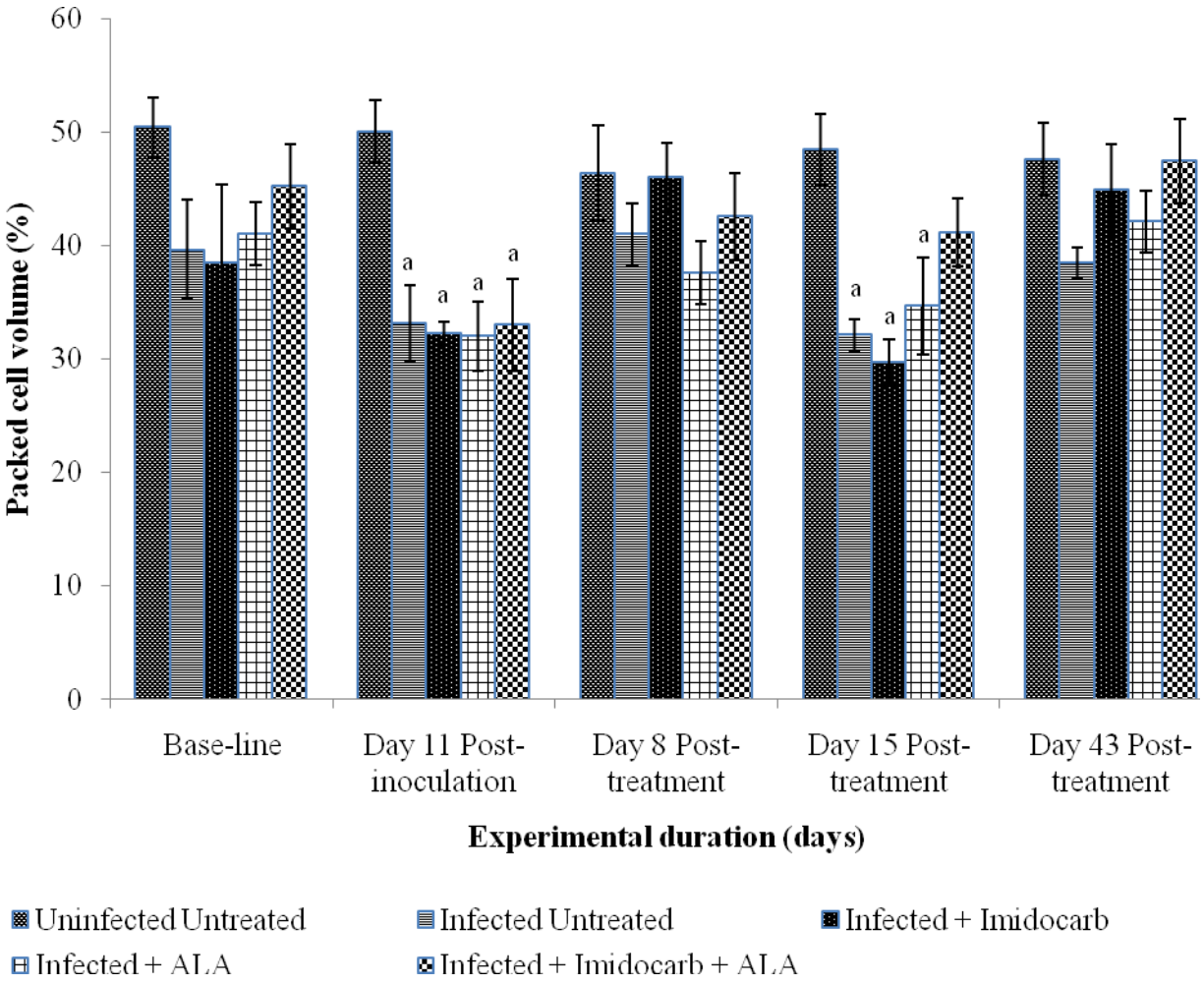


Figure 4.5: Effect of treatments on packed cell volume of dogs experimentally infected with *Babesia canis vogeli*

[a]P < 0.05 versus uninfected untreated group

Normal range: 37 - 55%

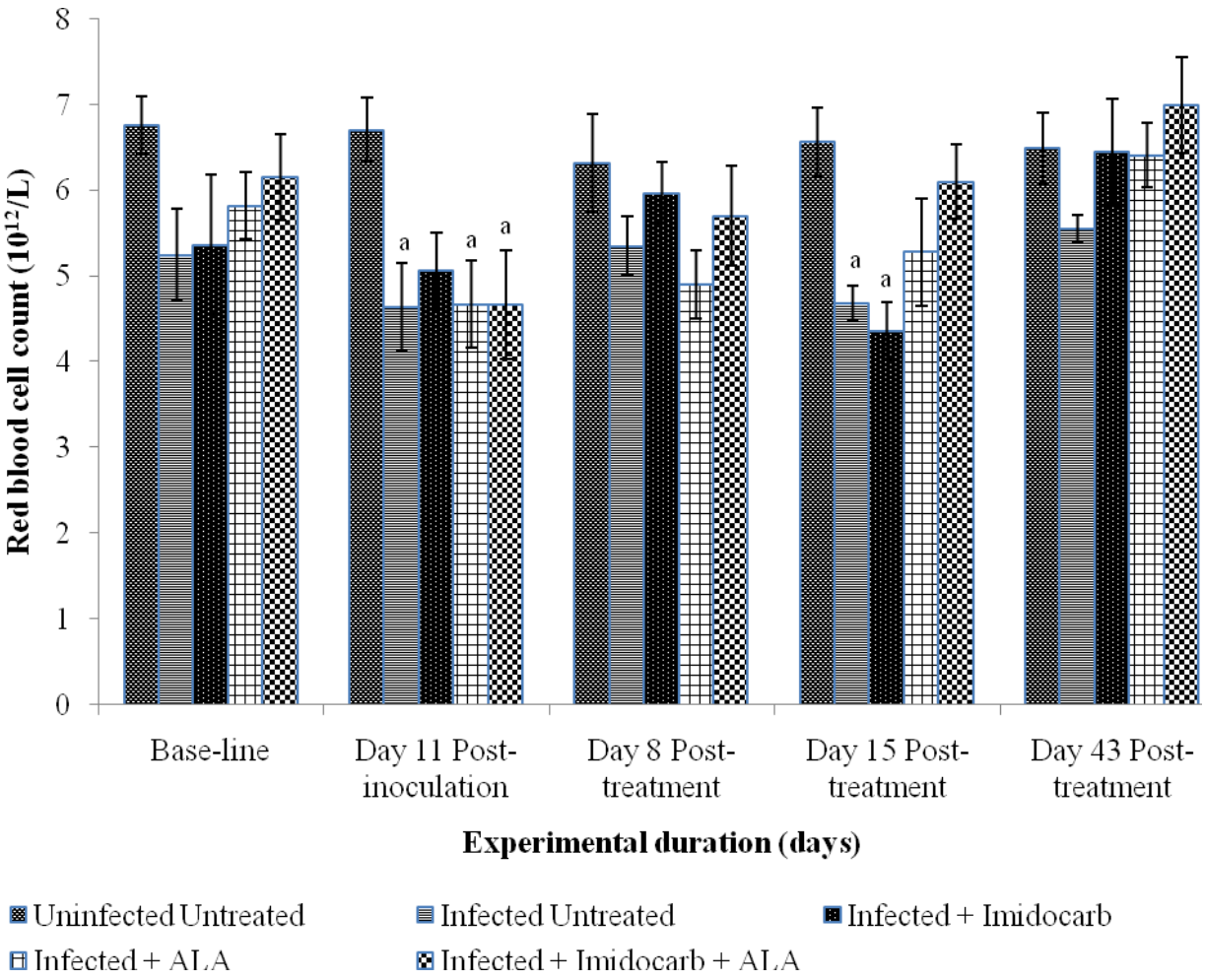


Figure 4.6: Effect of treatments on red blood cell count of dogs infected with *Babesia canis vogeli*

[a]P < 0.05 versus uninfected untreated group

Normal range: 5.5 - 8.5 x 10^{12}/L

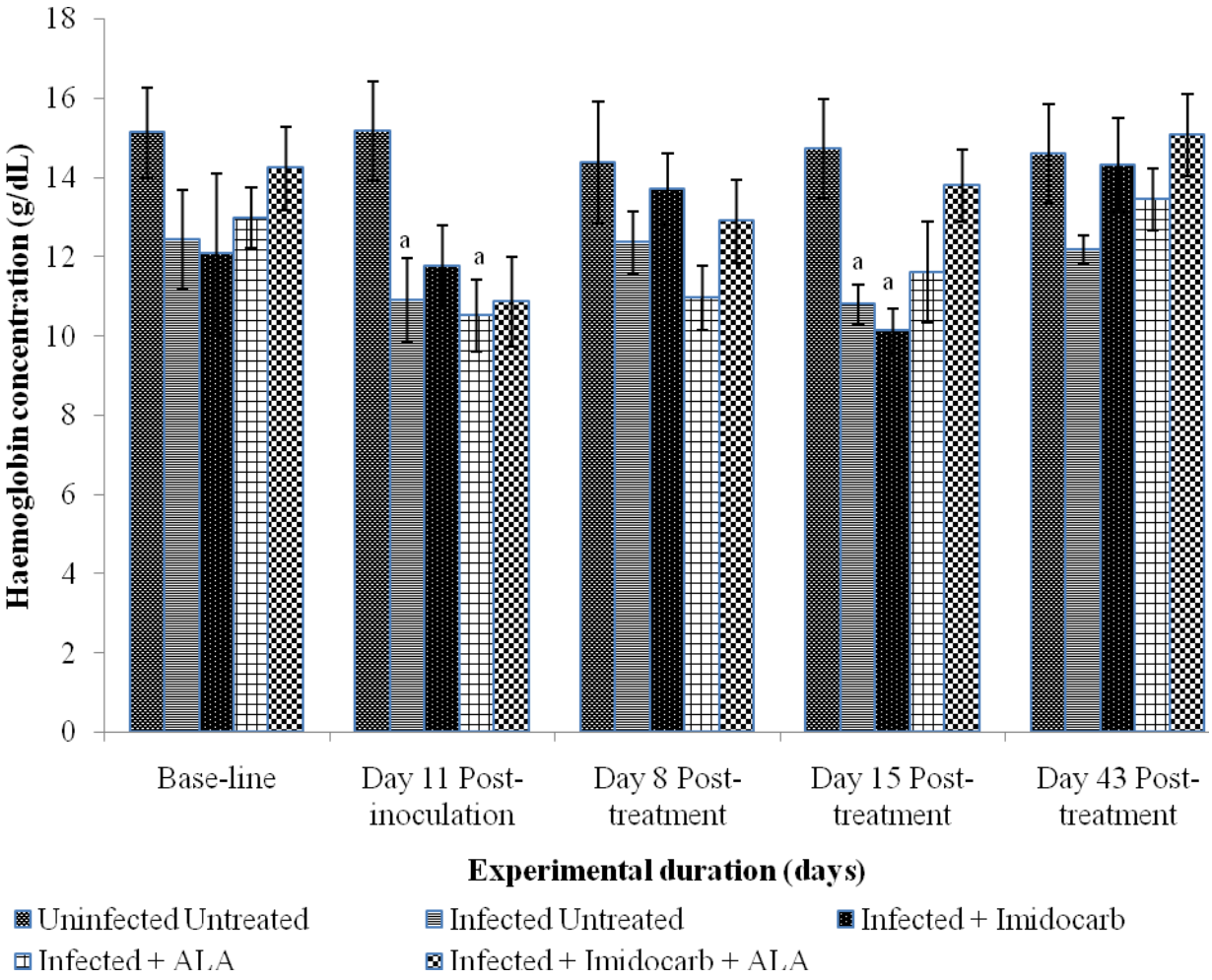


Figure 4.7: Effect of treatments on haemoglobin concentration of dogs infected with *Babesia canis vogeli*

[a]P < 0.05 versus uninfected untreated group

Normal range: 12 - 15 g/dL

4.5.4 Effect of treatments on erythrocyte indices

4.5.4.1 Effect of treatments on mean corpuscular volume

In the infected + imidocarb dogs, MCV (Figure 4.8) was higher ($P < 0.01$) at day 8 PT (77.00 ± 0.74 fl) compared to PT days 15 (68.20 ± 0.64 fl), and 43 (69.57 ± 0.84 fl; $P < 0.05$), respectively. In the infected + ALA dogs, there was a higher ($P < 0.05$) MCV at base-line (70.66 ± 1.50 fl) and day 8 PT (72.00 ± 0.85 fl; $P < 0.01$), compared to PT days 15 (65.58 ± 0.77 fl) and 43 (65.53 ± 0.69 fl), respectively. In the infected + imidocarb + ALA dogs, a higher ($P < 0.05$) MCV was recorded at day 8 PT (74.73 ± 1.63 fl) than at PT days 15 (67.80 ± 1.39 fl) and 43 (67.80 ± 1.50 fl), respectively.

At day 11 PT, MCV was significantly ($P < 0.01$) higher in the uninfected untreated dogs (74.60 ± 0.14 fl), compared to infected + ALA dogs (68.78 ± 1.29 fl). At day 8 PT, the MCV in the infected untreated dogs (76.50 ± 1.12 fl) and infected + imidocarb dogs (77.00 ± 0.74 fl) was each significantly ($P < 0.05$) higher than that of infected + ALA dogs (72.00 ± 0.85 fl). At day 15 PT, the MCV in uninfected untreated dogs (73.75 ± 0.50 fl) was higher ($P < 0.01$) compared to values recorded in the infected untreated (68.60 ± 0.80 fl), infected + imidocarb (68.20 ± 0.64 fl; $P < 0.05$), infected + ALA (65.58 ± 0.77 fl; $P < 0.001$) and infected + imidocarb + ALA (67.80 ± 1.50 fl; $P < 0.01$) dogs, respectively. At day 43 PT, the MCV in the uninfected untreated dogs (73.10 ± 0.84 fl) was higher ($P < 0.001$) higher than those of the infected + ALA dogs (65.58 ± 0.69 fl) and infected + imidocarb + ALA dogs (67.80 ± 1.50 fl; $P < 0.05$), respectively.

4.5.4.2 Effect of treatments on mean corpuscular haemoglobin

There was no significant ($P > 0.05$) change in the mean corpuscular haemoglobin (MCH) (Figure 4.9) within the uninfected untreated dogs. In the infected untreated dogs, MCH

recorded both at base-line (23.73 ± 0.37 pg) and day 11 PI (23.75 ± 0.38 pg) were higher than the value obtained at day 43 PT (21.95 ± 0.39 pg). There was no significant ($P > 0.05$) difference in MCH within the infected + imidocarb group at the different durations of the study. In the infected + ALA dogs, MCH at day 11 PI (33.10 ± 0.53 pg) was higher ($P < 0.05$) than day 43 PT (32.05 ± 0.32 pg).

4.5.4.3 Effect of treatments on mean corpuscular haemoglobin concentration

In the infected untreated dogs, the MCHC (Figure 4.10) was lower ($P < 0.05$) at base-line (31.67 ± 0.50 g/dL) compared to that of day 11 PI (33.10 ± 0.28 g/dL) or day 15 PT (33.77 ± 0.15 g/dL); but higher ($P < 0.05$) than at day 8 PT (30.18 ± 0.28 g.dL). The MCHC at day 43 PT (31.62 ± 0.37 g/dL) was lower ($P < 0.05$) compared to days 11 PI (33.10 ± 0.28 g/dL) and days 15 PT (33.77 ± 0.15 g/dL; $P < 0.01$), but higher than the value obtained at day 8 (30.18 ± 0.28 g/dL; $P < 0.05$) and 43 (31.62 ± 0.37 g/dL; $P < 0.01$), compared to the value obtained at PT day. In the infected + imidocarb dogs, the base-line MCHC (31.60 ± 0.53 g/dL) was lower ($P < 0.05$) compared to that of day 15 PT (34.17 ± 0.48 g/dL). There was a higher ($P < 0.05$) MCHC at day 11 PI (32.50 ± 0.60 g/dL) compared to the value recorded at day 8 PT (29.87 ± 0.38 g/dL). A higher ($P < 0.05$) MCHC was also recorded at day 15 PT (34.17 ± 0.48 g/dL) compared to base-line (31.60 ± 0.53 g/dL); PT days 8 (29.87 ± 0.38 g/dL; $P < 0.001$) and 43 (32.00 ± 0.27 g/dL), respectively. In the infected + ALA dogs, the MCHC at base-line (31.66 ± 0.36 g/dL) was lower than that of day 15 PT (33.72 ± 0.43 g/dL). A significantly ($P < 0.05$) lower MCHC was recorded at day 8 PT (31.16 ± 0.47 g/dL) compared to days 11 PI (33.10 ± 0.53 g/dL) and 15 PT (33.72 ± 0.43 g/dL; $P < 0.01$), respectively. In the infected + imidocarb + ALA dogs, MCHC at day 8 PT (30.40 ± 0.25 g/dL) was lower ($P < 0.01$) than that recorded at day 11 PI (33.23 ± 0.77 g/dL) or day 15 PT (33.60 ± 0.35 g/dL), respectively.

The MCHC was lower ($P < 0.001$) at day 11 PI, in the uninfected untreated dogs (30.18 ± 0.80 g/dL), compared to those of the infected untreated dogs (33.10 ± 0.28 g/dL); infected + imidocarb dogs (32.50 ± 0.60 g/dL; $P < 0.05$); infected + ALA dogs (33.10 ± 0.53 g/dL; $P < 0.001$), and infected + imidocarb + ALA dogs (33.23 ± 0.77 g/dL; $P < 0.001$), respectively. At day 8 PT, variations between the different groups were insignificant. At day 15 PT, there was a lower ($P < 0.001$) MCHC in the uninfected untreated dogs (30.30 ± 0.71 g/dL), compared to infected untreated dogs (33.77 ± 0.15 g/dL); infected + imidocarb dogs (34.17 ± 0.48 g/dL); infected + ALA dogs (33.72 ± 0.43 g/dL); and infected + imidocarb + ALA dogs (33.60 ± 0.35 g/dL), respectively. At day 43 PT, the difference in the MCHC values between the groups were insignificant.

4.5.5 Effect of treatments on total white blood cell count

There was no significant ($P > 0.05$) difference in the WBC (Figure 4.11) within the uninfected untreated and infected untreated groups at base-line, days 11 PI, 8 PT, 15 PT and 43 PT, respectively. In the infected + imidocarb dogs, a higher ($P < 0.001$) WBC count was recorded at base-line (26.43 ± 1.29 $x10^3/\mu L$), compared to days 11 PI (9.47 ± 1.28 $10^3/\mu L$); 8 PT (10.30 ± 0.82 $10^3/\mu L$); 15 PT (9.03 ± 0.94 $10^3/\mu L$); and 43 PT (7.00 ± 0.85 $10^3/\mu L$), respectively. At base-line, WBC count in the infected + imidocarb dogs (26.43 ± 1.29 $10^3/\mu L$) was significantly ($P < 0.001$) higher than in the infected untreated dogs (13.38 ± 1.09 $10^3/\mu L$); infected + ALA dogs (12.50 ± 1.73 $10^3/\mu L$); and infected + imidocarb + ALA (11.87 ± 1.68 $10^3/\mu L$) dogs, respectively. At days 11 PI, 8 PT and 15 PT, there was no significant ($P > 0.05$) difference in WBC counts between the groups. At day 43 PT, the WBC count in the uninfected untreated dogs (17.40 ± 2.66 $10^3/\mu L$) was significantly ($P < 0.05$) higher than infected + imidocarb (7.00 ± 0.85 $10^3/\mu L$) and infected + ALA dogs (8.48 ± 0.87 $10^3/\mu L$), respectively.

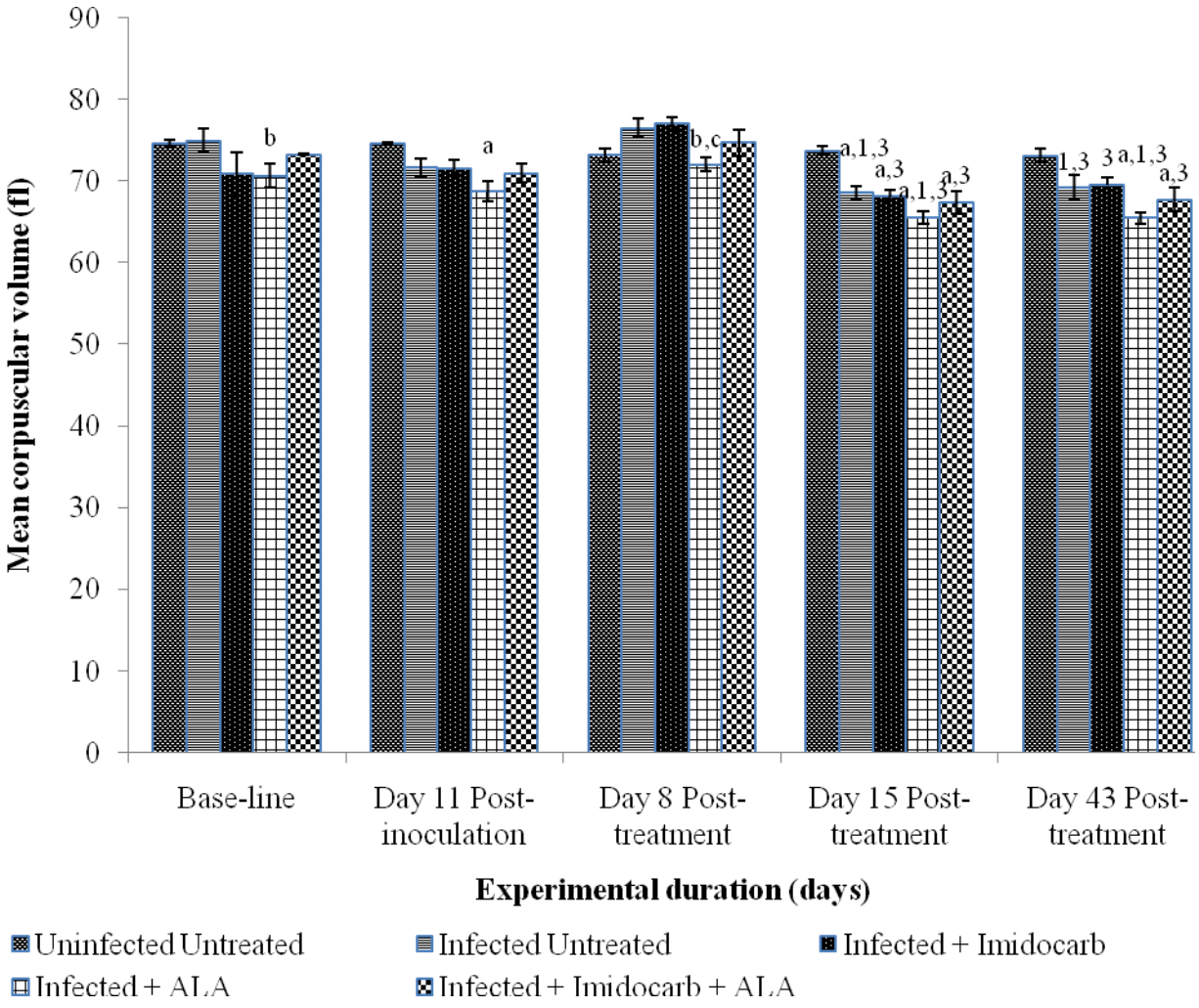


Figure 4.8: Effect of treatments on mean corpuscular volume of dogs experimentally infected with *Babesia canis vogeli*

[1,3]P < 0.05 versus base-line and 8 PT respectively
[a,b,c]P < 0.05 versus uninfected untreated, infected untreated and infected + imidocarb groups, respectively

Normal range: 64 - 73 fl

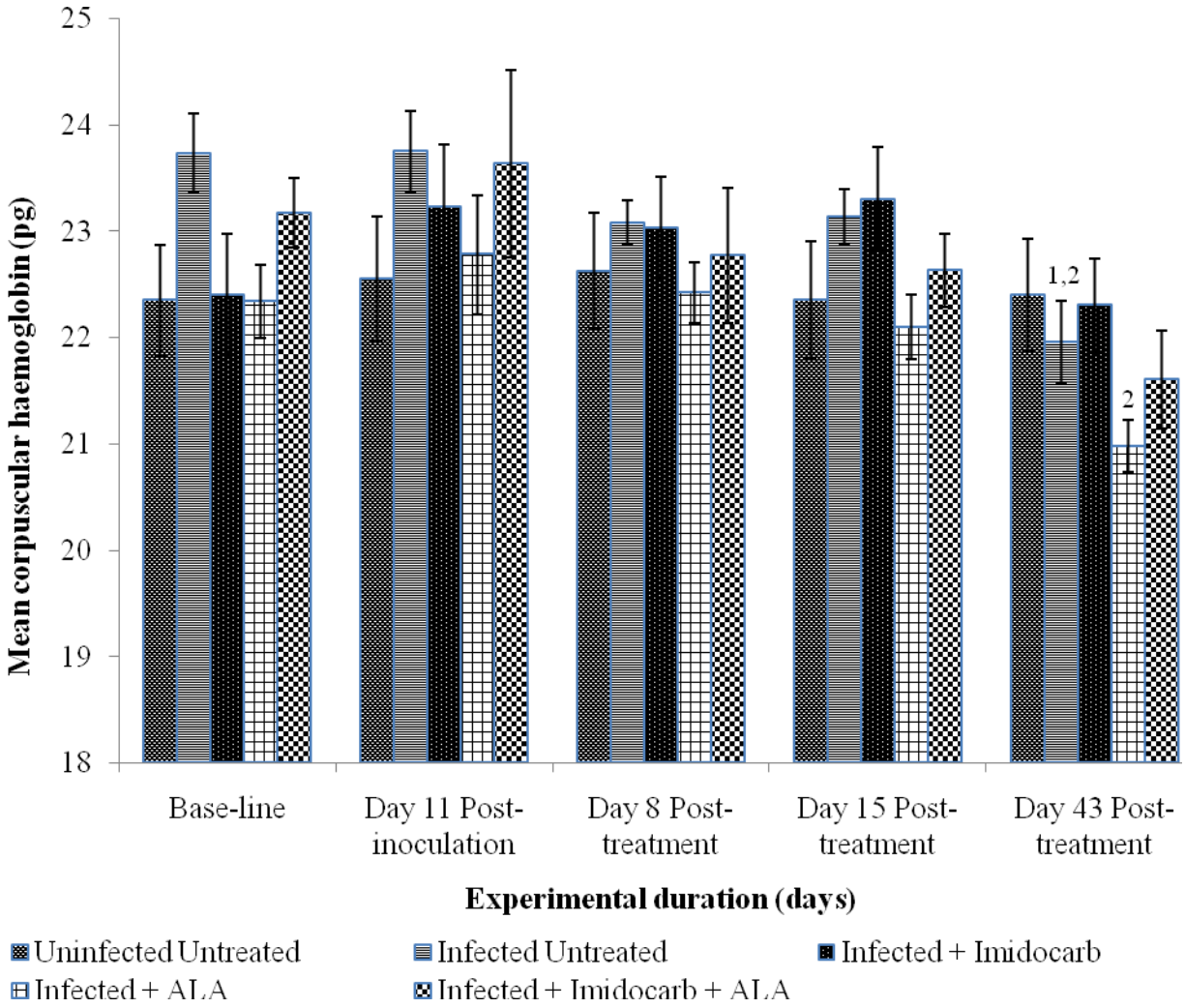


Figure 4.9: Effect of treatments on mean corpuscular haemoglobin of dogs experimentally infected with *Babesia canis vogeli*

[1,2]P < 0.05 versus base-line and day 11 PI respectively

Normal range: 22 - 26 pg

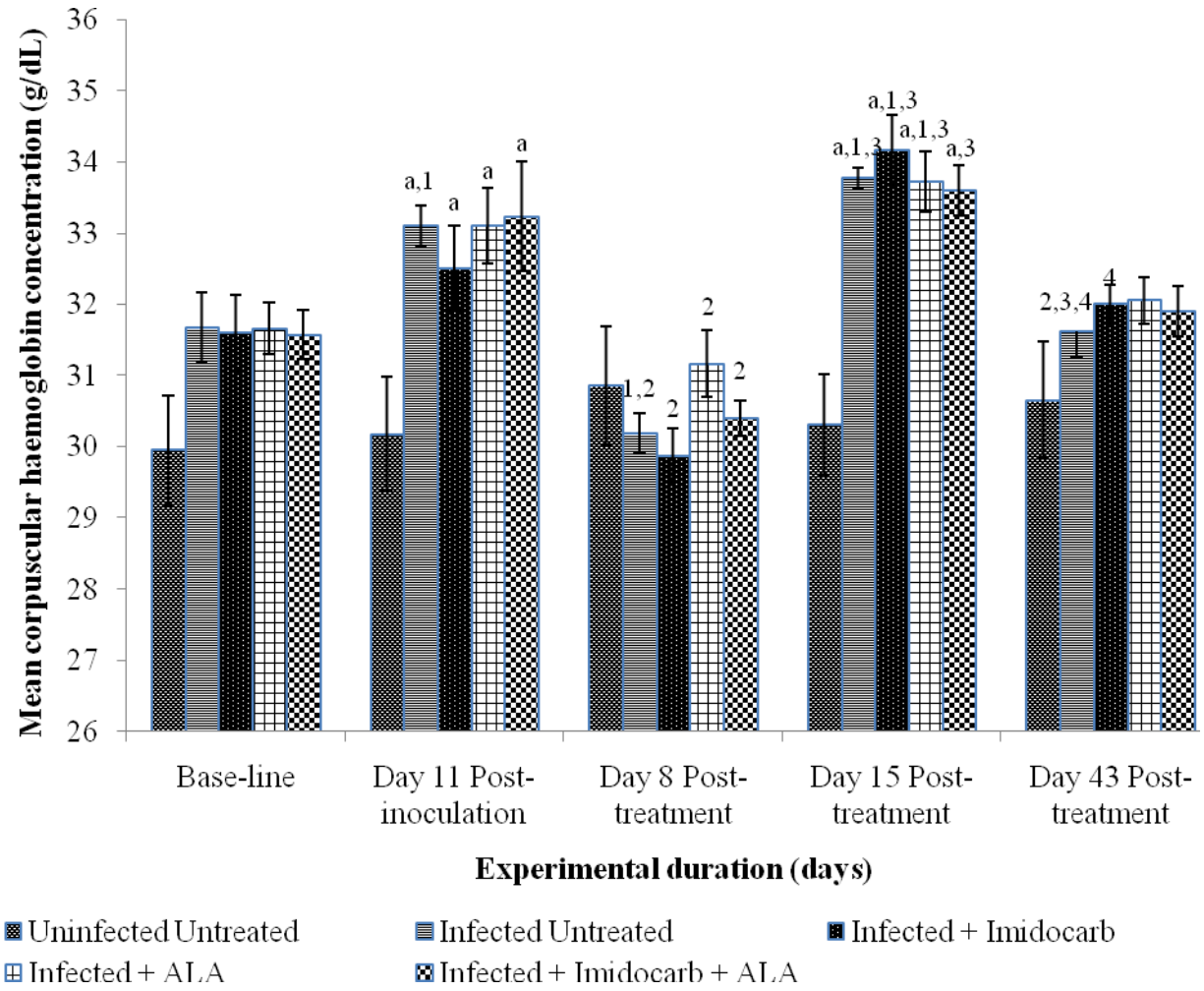


Figure 4.10: Effect of treatments on mean corpuscular haemoglobin concentration of dogs experimentally infected with *Babesia canis vogeli*

[1,2,3,4]P < 0.05 versus base-line, days 11 PI, 8 PT, 15 PT and 43 PT, respectively
[a]P < 0.05 versus uninfected untreated group

Normal range: 33 - 36 g/dL

4.5.5.1 Effect of treatments on neutrophil count

There was no significant ($P > 0.05$) difference in the neutrophil count (Figure 4.12) in dogs within the uninfected untreated, infected + imidocarb and infected + imidocarb + ALA groups. However, the infected untreated dogs had a higher ($P < 0.05$) neutrophil count at day 11 PI ($2.88 \pm 0.399\ 10^3/\mu L$), compared to corresponding values obtained at PT days 15 ($0.95 \pm 0.10\ 10^3/\mu L$) and 43 ($0.87 \pm 0.22\ 10^3/\mu L$). Similarly, infected + ALA dogs demonstrated a higher ($P < 0.01$) neutrophil count at day 11 PI ($2.36 \pm 0.38\ 10^3/\mu L$) than at day 43 PT ($0.60 \pm 0.12\ 10^3/\mu L$). There was no significant difference in the neutrohil count at base-line, days 11 PI, 8 PT, 15 PT, respectively, between the groups. However, a higher ($P < 0.05$) difference was recorded in the uninfected untreated group ($2.18 \pm 0.32\ 10^3/\mu L$), compared to the infected untreated ($0.87 \pm 0.22\ 10^3/\mu L$) and infected + ALA ($0.60 \pm 0.12\ 10^3/\mu L$) dogs at day 43 PT.

4.5.5.2 Effect of treatments on lymphocyte count

There was no significant ($P > 0.05$) difference in the lymphocyte count (Figure 4.13) within uninfected untreated and infected + ALA groups. The infected + imidocarb dogs had a higher ($P < 0.001$) lymphocyte count at base-line ($16.87 \pm 0.86\ 10^3/\mu L$), compared to days 11 PI ($5.27 \pm 0.74\ 10^3/\mu L$); 8 PT ($7.27 \pm 0.12\ 10^3/\mu L$); 15 PT ($6.30 \pm 0.49\ 10^3/\mu L$) and 43 PT ($5.77 \pm 0.49\ 10^3/\mu L$), respectively. The infected + imidocarb + ALA dogs had a higher ($P < 0.05$) lymphocyte count at base-line ($8.43 \pm 1.53\ 10^3/\mu L$); days 8 PT ($8.37 \pm 1.04\ 10^3/\mu L$); 15 PT ($8.63 \pm 2.05\ 10^3/\mu L$); compared to day 43 PT ($7.20 \pm 1.03\ 10^3/\mu L$), respectively. At day 11 PI, there was a significantly ($P < 0.05$) higher lymphocyte count in the uninfected untreated dogs ($12.15 \pm 2.00\ 10^3/\mu L$) compared to infected + imidocarb dogs ($5.27 \pm 0.74\ 10^3/\mu L$). There was no significant ($P > 0.05$) difference at PT days 8 and 15

between the groups. At day 43 PT, uninfected untreated dogs (12.60 ± 2.13 10^3/µL) had a higher ($P < 0.05$) lymphocyte count than infected + imidocarb dogs (5.77 ± 0.49 10^3/µL).

4.5.5.3 Effect of treatments on neutrophil:lymphocyte ratio

The neutrophil:lymphocyte ratio (NLR) (Figure 4.14) at day 11 PI in the uninfected untreated dogs (0.19 ± 0.02) was lower ($P < 0.05$) compared to the infected untreated dogs (0.36 ± 0.09); infected + imidocarb dogs (0.27 ± 0.05; $P < 0.01$); infected + ALA dogs (0.34 ± 0.06; $P < 0.05$); and infected + imidocarb + ALA dogs (0.29 ± 0.08; $P < 0.05$), respectively. Similarly, at day 8 PT, higher ($P < 0.001$) NLR ration was recorded in the uninfected untreated dogs (0.18 ± 0.04) compared to infected untreated dogs (0.16 ± 0.03); infected + imidocarb (0.17 ± 0.02); infected + ALA dogs (0.17 ± 0.02), but lower ($P < 0.001$) NLR ratio compared to infected + imidocarb +ALA (0.19 ± 0.07), respectively. At days 15 and 43 PT, there was no significant ($P > 0.05$) difference between the groups.

4.5.6 Effect of treatments on platelet counts

There was no significant ($P > 0.05$) difference in the platelet count (Figure 4.15) within and between the different groups; uninfected untreated, infected untreated, infected + imidocarb, infected + ALA and infected + imidocarb + ALA at base-line, days 11 PI, 8 PT, 15 PT and 43 PT, respectively. Although in all the infected groups, there was marked decrease in the platelet counts at day 11 PI compared to the base-line.

4.5.7 Effect of treatments on glucose concentration

The glucose concentration (Figure 4.16) in the infected untreated dogs, at day 15 PT (5.18 ± 0.13 mmol/L) was significantly ($P < 0.01$) higher than at day 43 PT (3.93 ± 0.26 mmol/L). There was no significant ($P > 0.05$) difference in glucose concentration in dogs in

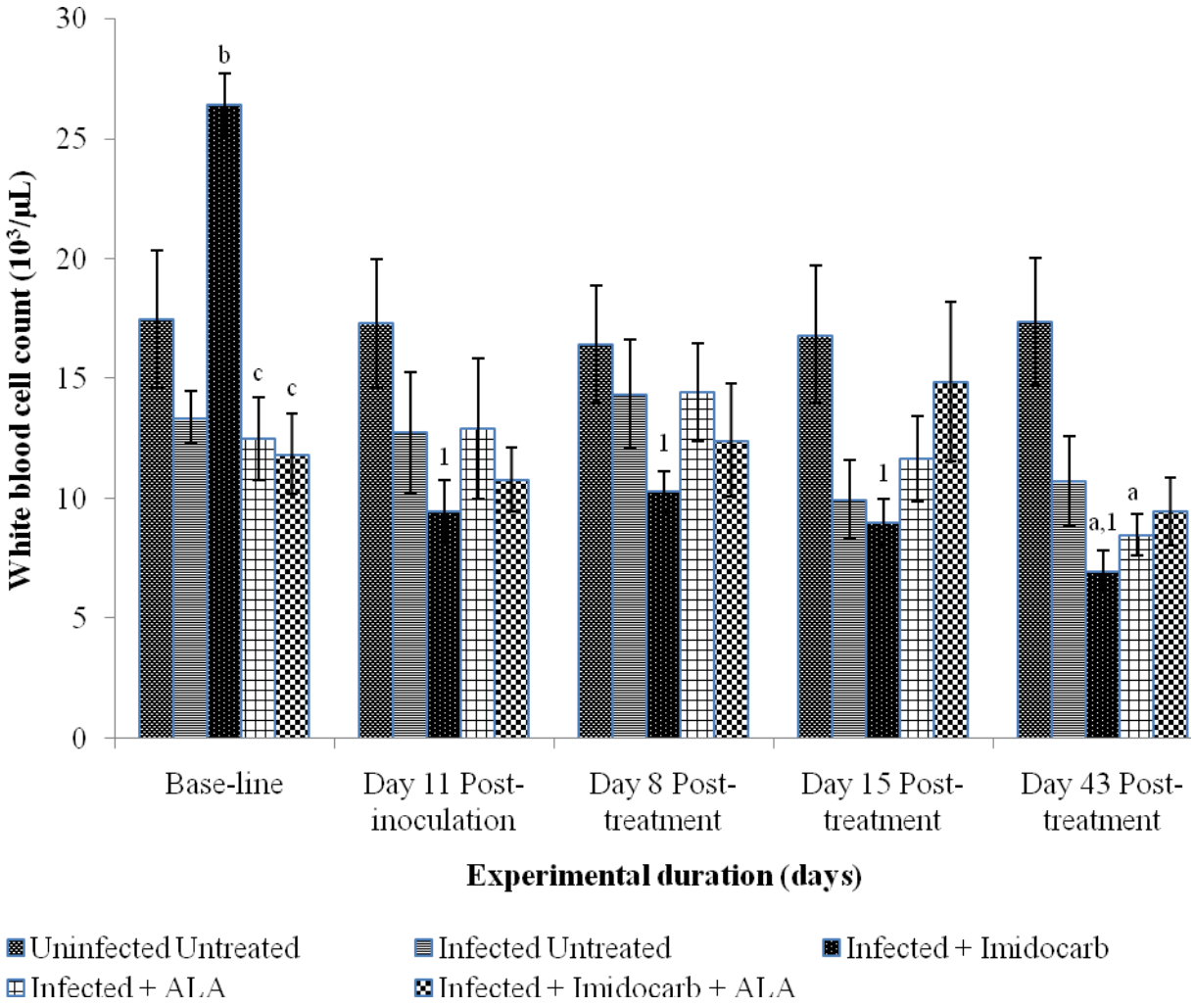


Figure 4.11: Effect of treatments on white blood cell count of dogs experimentally infected with *Babesia canis vogeli*

[1]P < 0.05 versus base-line
[a,b,c]P < 0.05 versus uninfected untreated, infected untreated and infected + imidocarb groups, respectively

Normal range: 6 - 17 x 10^3/µL

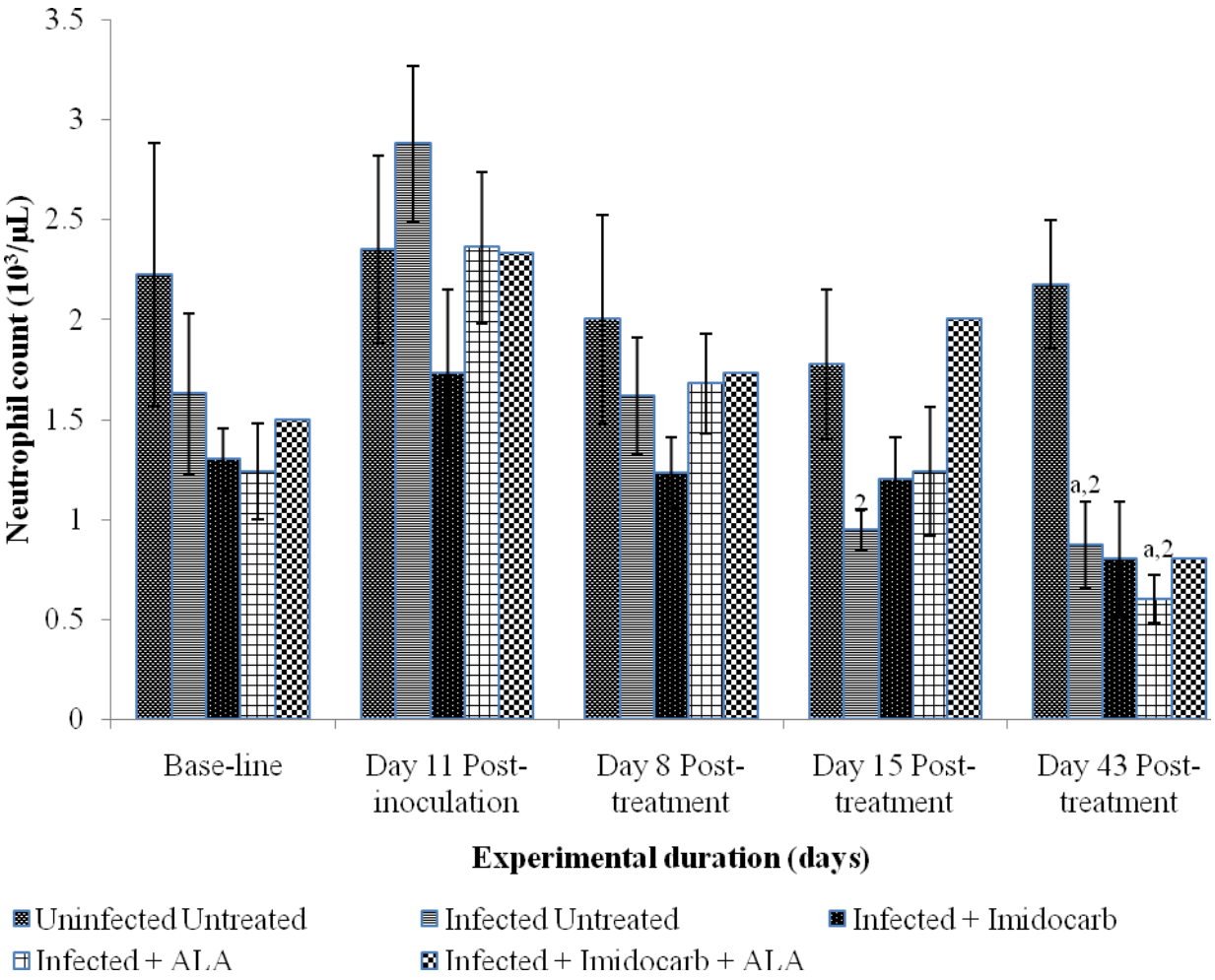


Figure 4.12: Effect of treatments on neutrophil count of dogs experimentally infected with *Babesia canis vogeli*

[2]P < 0.05 versus day 11 PI
[a]P < 0.05 versus uninfected untreated group

Normal range: 3 - 11.5 x10^3/µL

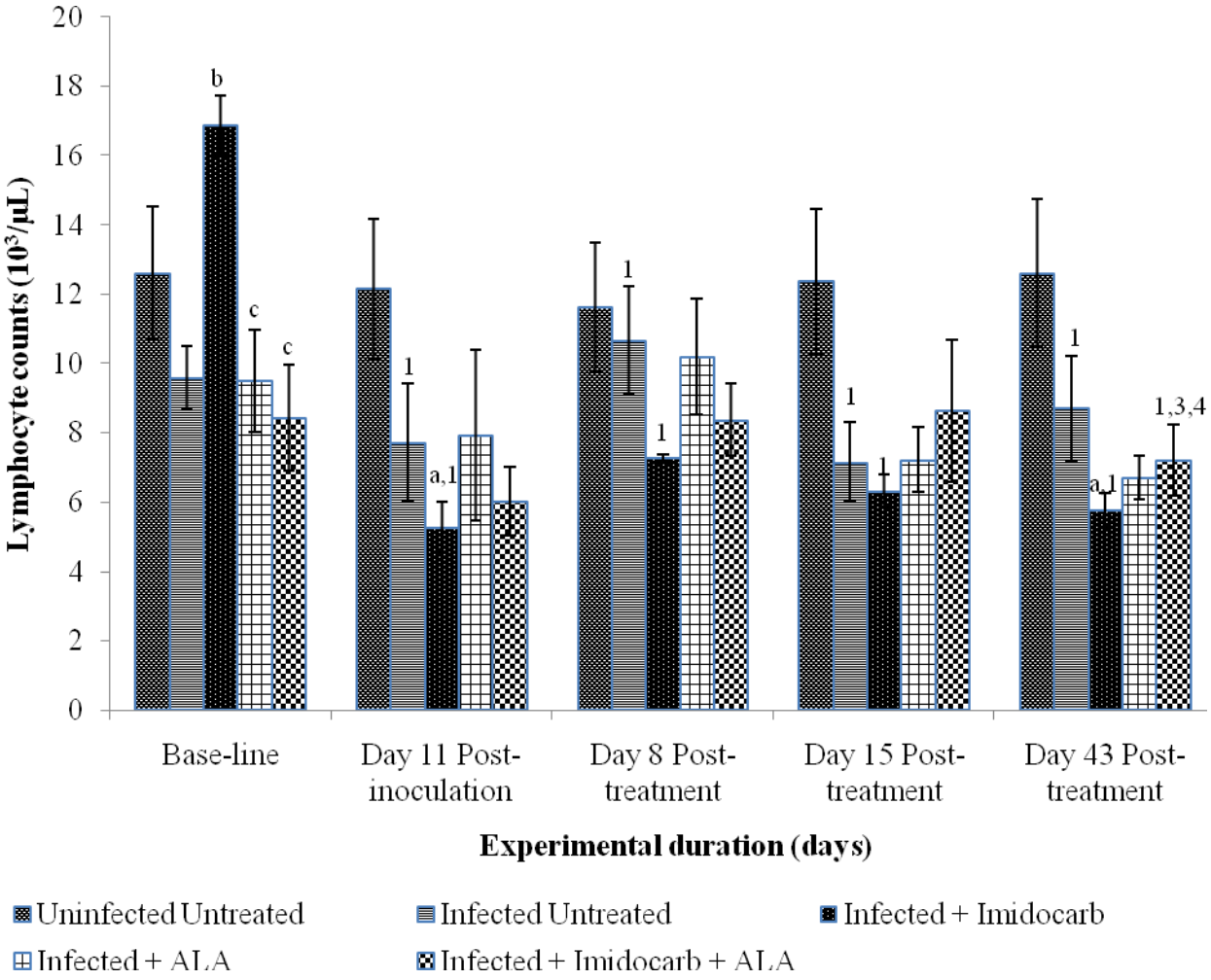


Figure 4.13: Effect of treatments on lymphocyte count of dogs experimentally infected with *Babesia canis vogeli*

[1,3,4]P < 0.05 versus base-line, days 8 PTand 15 PT, respectively
[a,b,c]P < 0.05 versus uninfected untreated, infected untreated and infected + imidocarb groups, respectively

Normal range: 1 - 4 x 10^3/µL

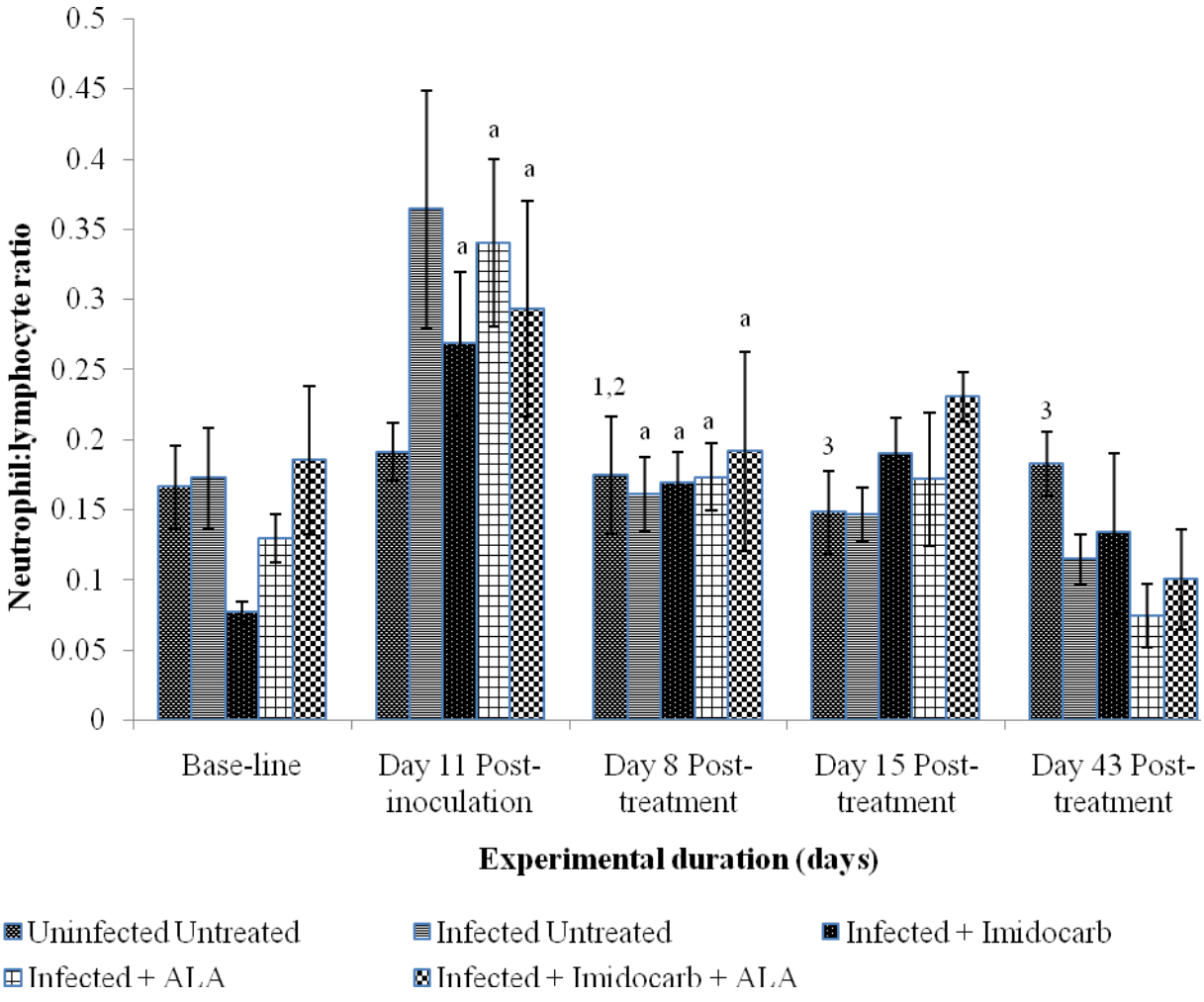


Figure 4.14: Effect of treatments on neutrophil:lymphocyte ratio of dogs experimentally infected with *Babesia canis vogeli*

[1,2,3]P < 0.05 versus base-line, days 11 PI and 8 PT, respectively
[a]P < 0.05 versus uninfected untreated group

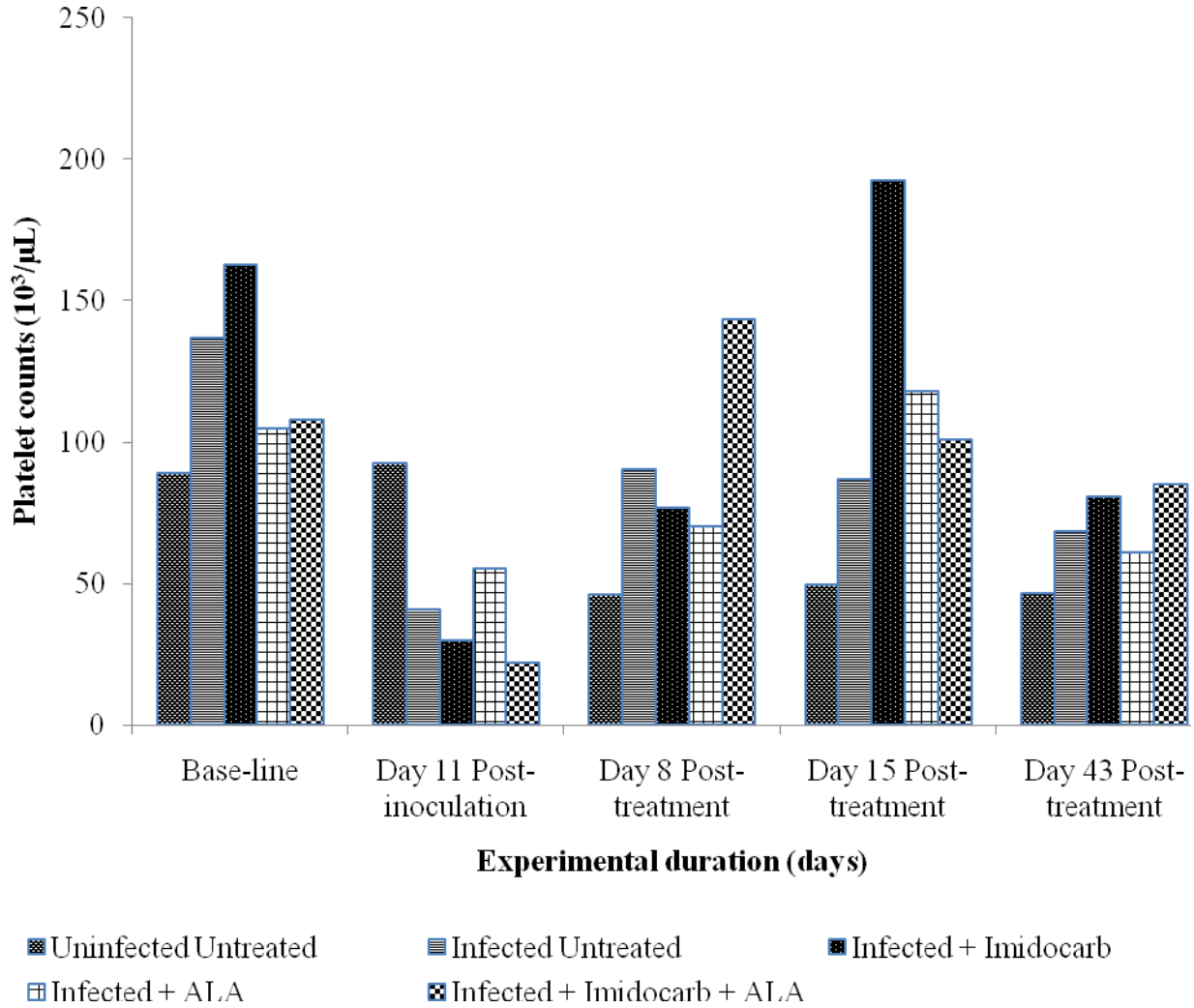


Figure 4.15: Effect of treatments on platelet count of dogs experimentally infected with *Babesia canis vogeli*

Normal range: 150 - 400 x10^3/µL

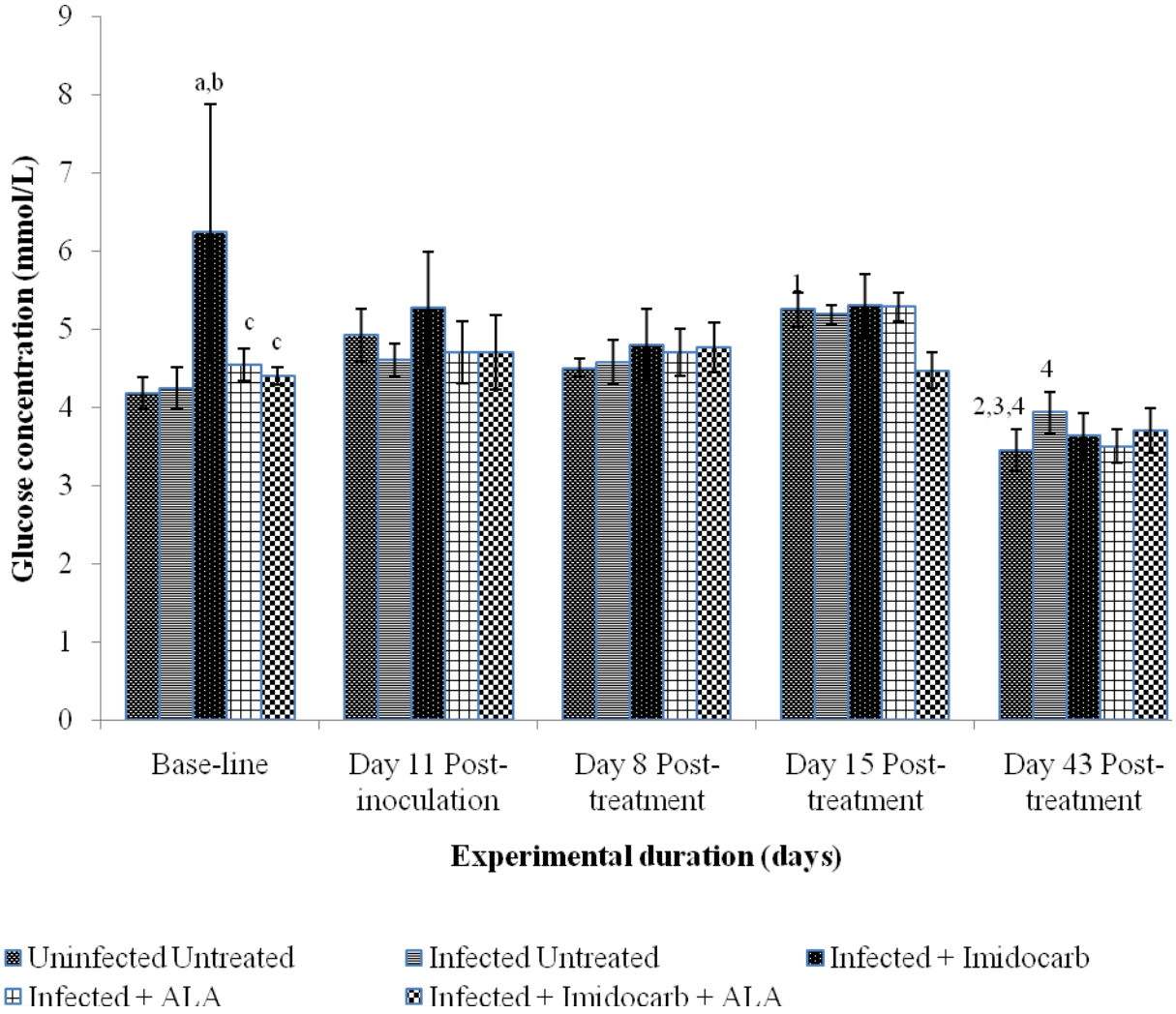


Figure 4.16: Effect of treatments on glucose concentration of dogs experimentally infected with *Babesia canis vogeli*

[1,2,3,4]P < 0.05 versus base-line, days 11 PI, 8 PT and 43 PT, respectively
[a,b,c,]P < 0.05 versus uninfected untreated, infected untreated and infected + imidocarb groups, respectively

Normal range: 3.3 - 5.5 mmol/L

the infected + imidocarb, infected + ALA and infected + imidocarb + ALA groups. The glucose concentrations were all within the normal range of 3.3 - 5.5 mmol/L.

4.5.8 Effect of treatments on erythrocyte osmotic fragility

4.5.8.1 Effect of treatments at base-line

The percentage haemolysis increased with decreasing NaCl concentrations (Figure 4.17). At 0.5% NaCl concentration, the percentage haemolysis in the uninfected untreated dogs (51.88 ± 0.22%) and infected untreated dogs (51.71 ± 0.38%) was lower ($P < 0.05$) than the infected + imidocarb dogs (55.34 ± 0.37%). At 0.3% NaCl concentration, the percentage haemolysis of dogs in the infected + imidocarb group (75.31 ± 0.38%) was significantly ($P < 0.05$) higher than dogs in the uninfected untreated (72.21 ± 0.61%); and infected + imidocarb + ALA (72.33 ± 1.86%) groups, respectively.

4.5.8.2 Effect of treatments at day 11 post-inoculation

The percentage haemolysis increased with decreasing NaCl concentrations (Figure 4.18). At 0.7% NaCl concentration, the percentage haemolysis in the uninfected untreated dogs (5.67 ± 0.48 %) was lower ($P < 0.001$) than that recorded in the infected untreated dogs (53.50 ± 0.74%); infected + imidocarb dogs (49.24 ± 5.74%); infected + ALA dogs (31.24 ± 2.31%); and infected + imidocarb + ALA dogs (24.12 ± 2.07%). The percentage haemolysis in the infected untreated dogs (53.50 ± 0.74%) was higher ($P < 0.001$) compared to those of the infected + ALA dogs (31.24 ± 2.31%) and infected + imidocarb + ALA dogs (24.12 ± 2.07%). Higher ($P < 0.001$) percentage haemolysis was recorded in the infected + imidocarb dogs (49.24 ± 5.74%) compared to the infected + ALA dogs (31.24 ± 2.31%) and infected + imidocarb + ALA dogs (24.12 ± 2.07%).

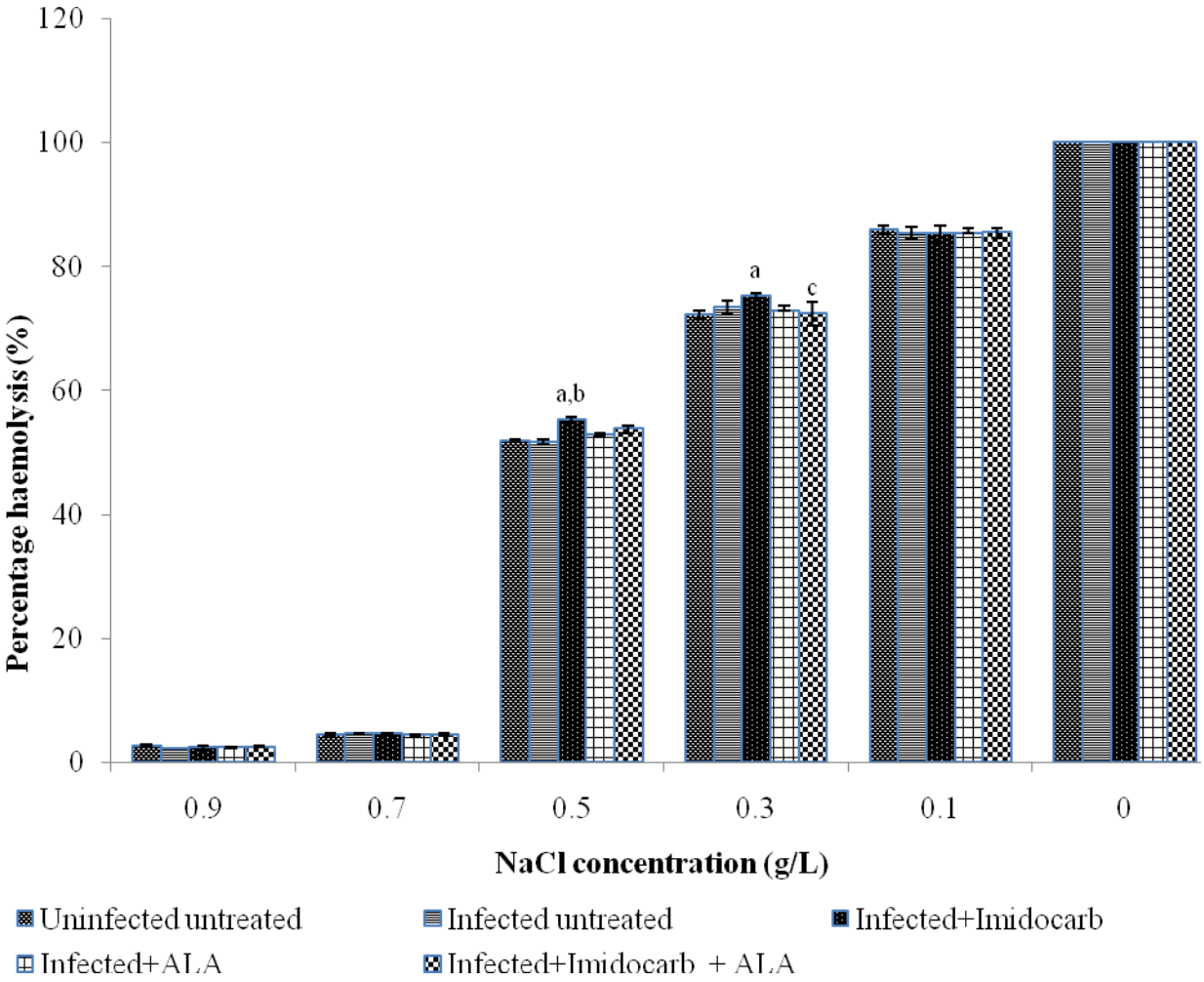


Figure 4.17: Effect of treatments on base-line erythrocyte osmotic fragility of Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli*

[a,b,c]P < 0.05 versus uninfected untreated, infected untreated and infected + imidocarb groups, respectively

At 0.5% NaCl concentration, the percentage haemolysis in the uninfected untreated dogs (39.81 ±2.08%) was lower ($P < 0.001$) than the infected untreated (74.90 ± 1.15%); infected + imidocarb dogs (67.41 ± 0.63%). Percentage haemolysis in the infected untreated dogs was significantly ($P < 0.001$) higher than that of the infected + ALA (39.28 ± 5.64%) and infected + imidocarb + ALA (47.21 ± 7.91%) dogs. In the infected + imidocarb dogs, the percentage haemolysis was higher ($P < 0.001$) than the infected + ALA dogs and infected + imidocarb + ALA dogs.

At 0.3% NaCl concentration, uninfected untreated dogs (73.99 ± 0.76%) was significantly ($P < 0.001$) lower than the infected untreated (98.30 ± 1.57%); infected + imidocarb (95.04 ± 4.80 ± 1.49%); infected + ALA (98.51 ± 1.49%); and infected + imidocarb + ALA (92.64 ± 2.68%) dogs, respectively.

At 0.1% NaCl concentration, uninfected untreated dogs (86.68 ± 0.60%) had a lower ($P < 0.001$) percentage haemolysis, compared to the corresponding values obtained in infected untreated (99.64 ± 0.27%); infected + imidocarb (98.30 ± 1.31%; $P < 0.01$); infected + ALA (100.00 ± 0.00%); and infected + imidocarb + ALA (96.84 ± 1.73%; $P < 0.05$) dogs, respectively.

4.5.8.3 Effect of treatments at day 8 post-treatment

The percentage haemolysis increased with decreasing NaCl concentrations (Figure 4.19). The percentage haemolysis at 0.7% NaCl concentration in the infected untreated dogs (38.68 ± 1.90%) was significantly ($P < 0.001$) higher than the values recorded in the uninfected untreated (5.23 ± 0.25%); infected + imidocarb (4.68 ± 0.49%); infected + ALA (5.40 ± 0.24%); and infected + imidocarb + ALA dogs (3.48 ± 0.06%), respectively.

At 0.5% NaCl concentration, the percentage haemolysis in the uninfected untreated dogs (52.75 ± 11.95%) was significantly ($P < 0.001$) lower than in the infected untreated dogs (81.14 ± 4.34%); infected + imidocarb (74.10 ± 1.20%); infected + ALA dogs (76.79 ± 1.21%), respectively. The infected untreated, infected + imidocarb and infected + ALA dogs had significantly ($P < 0.001$) higher percentage haemolysis than the infected + imidocarb + ALA dogs (56.45 ± 0.80%).

At 0.3% NaCl concentration, the percentage haemolysis in the uninfected untreated dogs (80.41 ± 6.57%) was significantly ($P < 0.05$) lower than in the infected + ALA (94.12 ± 3.61%) dogs.

4.5.8.4 Effect of treatments at day 15 post-treatment

The percentage haemolysis increased with decreasing NaCl concentrations (Figure 4.20). At 0.7% NaCl concentration, the infected untreated dogs (42.01 ± 1.31%) had a higher ($P < 0.001$) percentage haemolysis compared to the uninfected untreated (5.23 ± 0.25%); infected + imidocarb dogs (4.44 ± 0.22%); infected + ALA dogs (5.96 ± 0.48%); and infected + imidocarb + ALA dogs (4.80 ± 1.05%), respectively.

At 0.5% NaCl concentration, percentage haemolysis in the infected untreated dogs (86.31 ± 1.39%) was higher ($P < 0.001$) than the values recorded in the uninfected untreated dogs (40.25 ± 1.89%); infected + imidocarb dogs (56.12 ± 1.95%); infected + ALA dogs (78.81 ± 2.94%); and infected + imidocarb + ALA dogs (34.93 ± 0.37%; $P < 0.05$). The percentage haemolysis in the infected + ALA dogs (78.81 ± 2.94%) was higher ($P < 0.001$) than the infected + imidocarb dogs (56.12 ± 1.95%) and infected + imidocarb + ALA dogs (34.93 ± 0.37%).

At 0.3% NaCl concentration, the uninfected untreated dogs (73.55 ± 0.82%) had a lower ($P < 0.001$) percentage haemolysis compared to the infected untreated dogs (99.77 ± 0.23%) and infected + ALA dogs (98.26 ± 1.74%). Percentage haemolysis in the infected + imidocarb dogs (74.18 ± 0.22%) was significantly ($P < 0.001$) lower compared to the infected untreated dogs and infected + ALA dogs. The infected + imidocarb + ALA dogs (54.08 ± 1.49%) had a lower percentage haemolysis, compared to the corresponding percentage obtained in the uninfected untreated (73.55 ± 0.82%); infected untreated (99.77 ± 0.23%); infected + imidocarb (74.18 ± 0.22%) and infected + ALA (98.26 ± 1.74%) dogs, respectively.

The percentage haemolysis at 0.1% NaCl concentration in the uninfected untreated dogs (98.64 ± 1.36%) was higher ($P < 0.001$) than the corresponding values recorded in the infected + imidocarb dogs (85.63 ± 0.43%) and infected + imidocarb + ALA dogs (90.38 ± 0.61%), respectively. Similarly, higher ($P < 0.001$) percentage haemolysis was recorded in the infected untreated dogs (100.00 ± 0.00%), compared to the infected + imidocarb dogs (85.63 ± 0.43%); and infected + imidocarb + ALA dogs (90.38 ± 0.61%). The percentage haemolysis in the infected + ALA dogs (99.09 ± 0.91%) was significantly ($P < 0.001$) higher than the infected + imidocarb dogs (85.63 ± 0.43%) and infected + imidocarb + ALA dogs (90.38 ± 0.61%), respectively.

4.5.8.5 Effect of treatments at day 43 post-treatment

The percentage haemolysis increased with decreasing NaCl concentrations (Figure 4.21). At 0.5% NaCl concentration, the infected untreated dogs (68.76 ± 5.25%) had a higher ($P < 0.001$) percentage haemolysis compared to the uninfected untreated dogs (57.49 ±0.72%), infected + imidocarb (56.72 ± 1.00%) and infected + imidocarb + ALA (56.39 ± 2.36%).

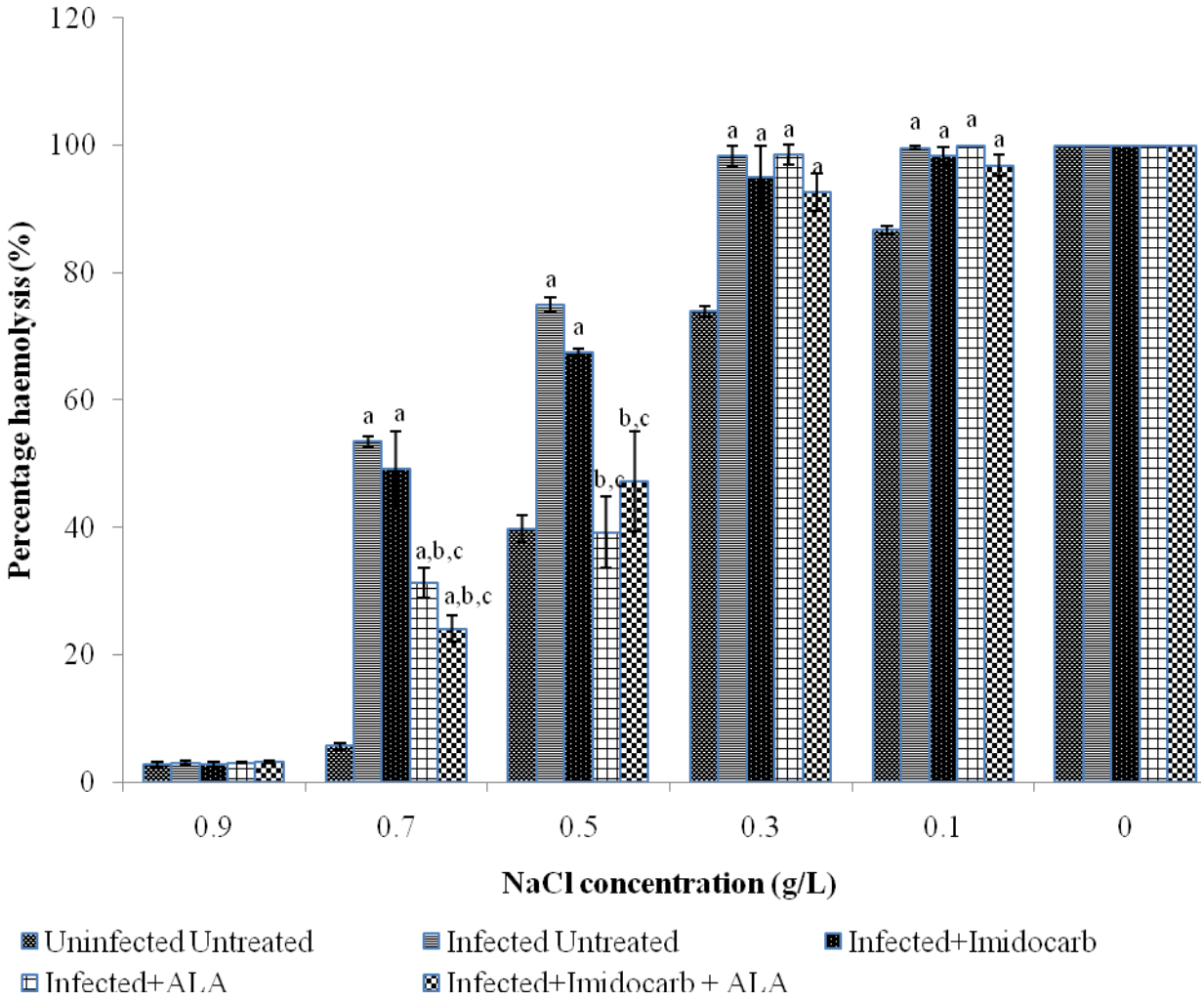


Figure 4.18: Effect of treatments on erythrocyte osmotic fragility of Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli* at day 11 PI

[a,b,c]P < 0.05 versus uninfected untreated, infected untreated and infected + imidocarb groups, respectively

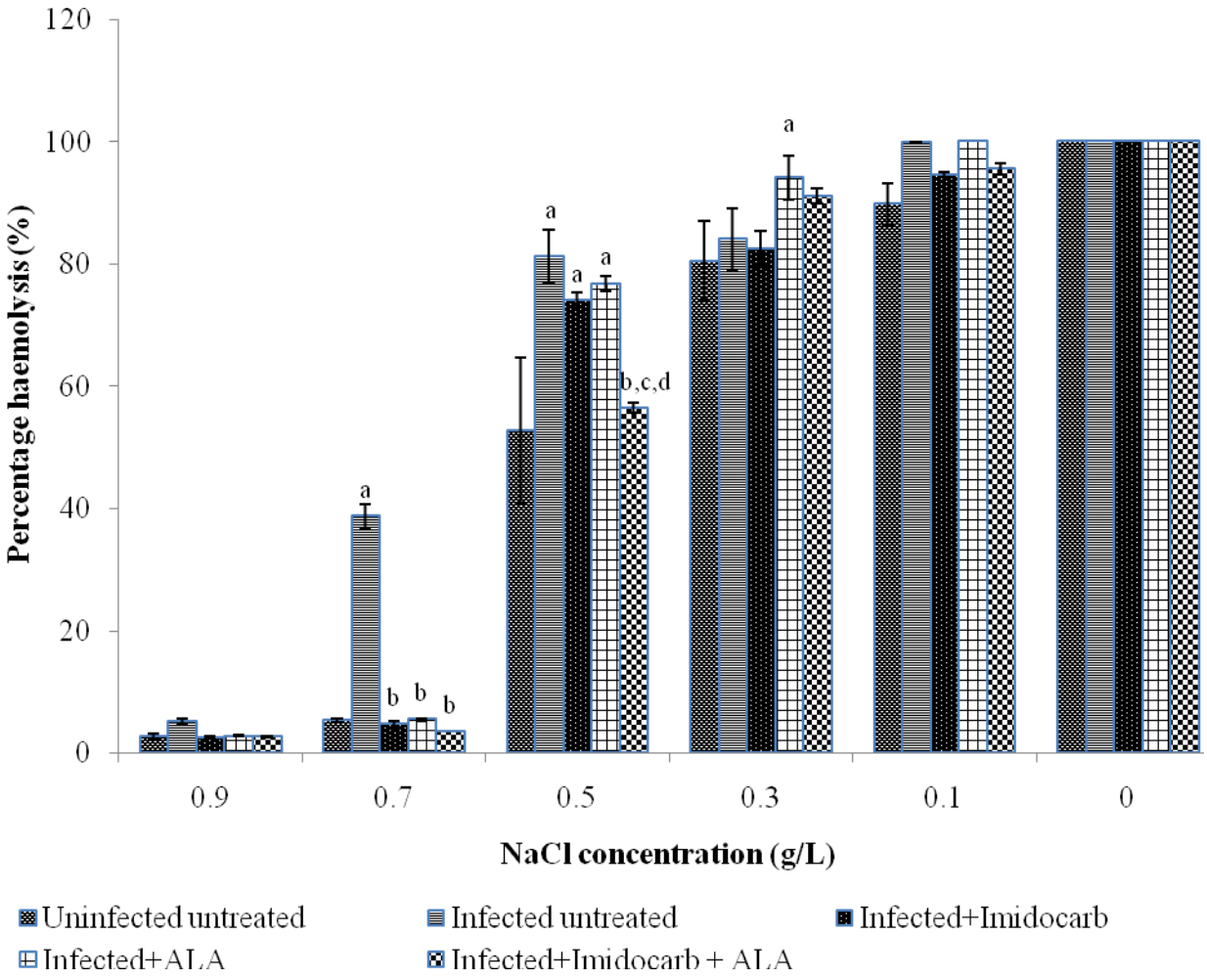


Figure 4.19: Effect of treatments on erythrocyte osmotic fragility of Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli* at day 8 PT

[a,b,c,d]P < 0.05 versus uninfected untreated, infected untreated, infected + imidocarb and infected + ALA groups, respectively

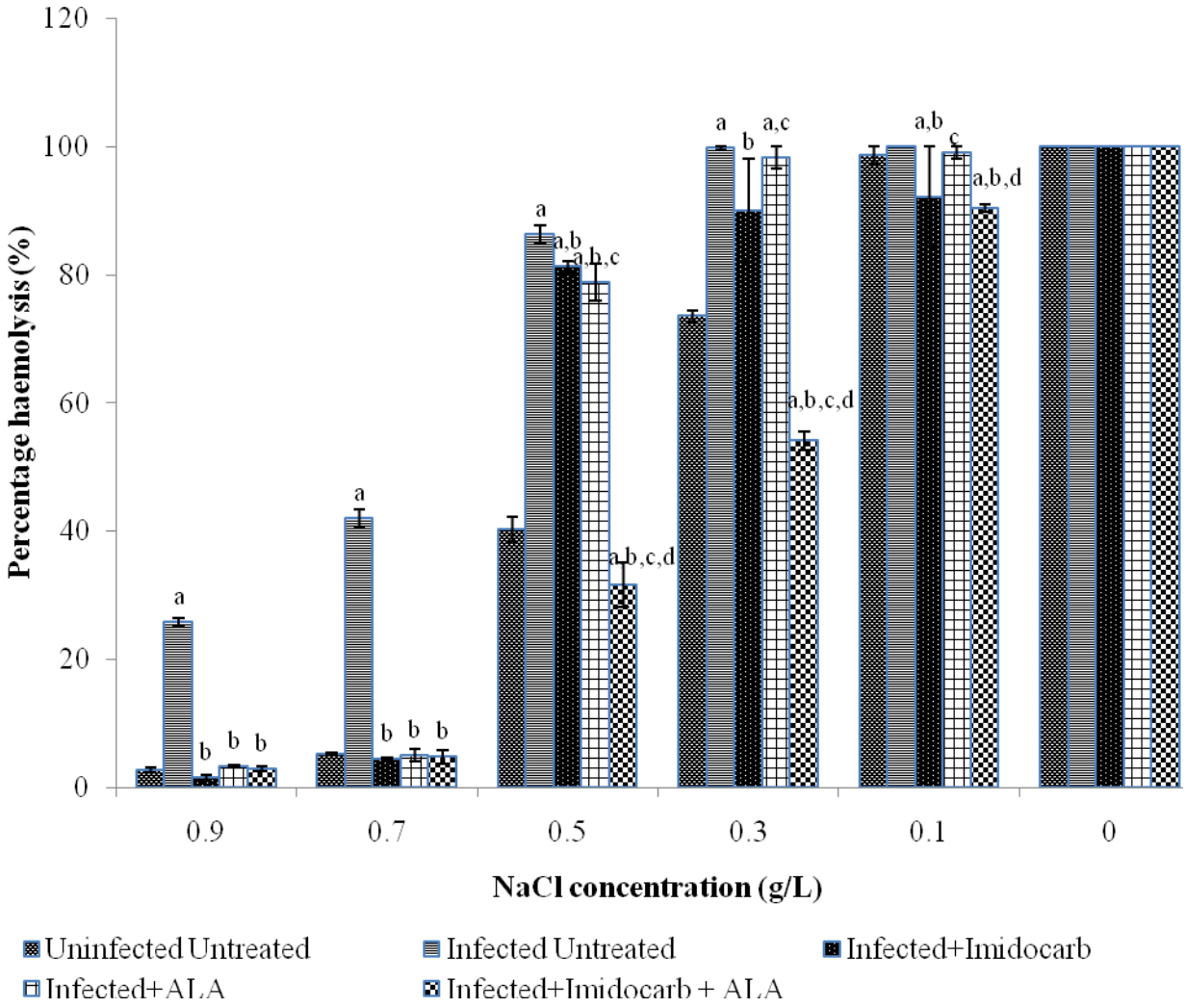


Figure 4.20: Effect of treatments on erythrocyte osmotic fragility of Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli* at day 15 PT

[a,b,c,d]P < 0.05 versus uninfected untreated, infected untreated, infected + imidocarb and infected + ALA groups, respectively

The infected + ALA dogs (65.70 ± 1.62%) had a higher ($P < 0.001$) percentage haemolysis compared to the infected + imidocarb + ALA dogs (56.39 ± 2.36%).

At 0.3% NaCl concentration, the percentage haemolysis in the uninfected untreated dogs (74.92 ± 1.39%) was lower ($P < 0.001$) compared to the infected untreated (85.72 ± 4.71%); infected + ALA (83.35 ± 0.47%) and infected + imidocarb + ALA (84.78 ± 0.15%) dogs, respectively.

At 0.1% NaCl concentration, the percentage haemolysis in the infected + imidocarb (88.76 ± 0.38%) and infected + imidocarb + ALA (81.69 ± 0.98%) was significantly ($P < 0.001$) lower than that of the uninfected untreated dogs (100.00 ± 0.00%) and infected untreated dogs (100.00 ± 0.00%).

4.6 Effect of Treatments on Serum Biochemical Parameters

4.6.1 Effect of treatments on serum electrolyte

4.6.1.1 Effect of treatments on serum potassium concentration

There was no significant ($P > 0.05$) difference in the potassium (K^+) concentration (Figure 4.22) within the groups at base-line, days 11 PI, 8 PT, 15 PT and 43 PT, respectively. There was no significant ($P > 0.05$) difference in the K^+ concentration at days 11 PI and 8 PT between the groups. At day 15 PT, K^+ concentration was lower ($P < 0.01$) in the uninfected untreated dogs (0.50 ± 0.03 mmol/L), compared to that of the infected untreated dogs (1.27 ± 0.11 mmol/).

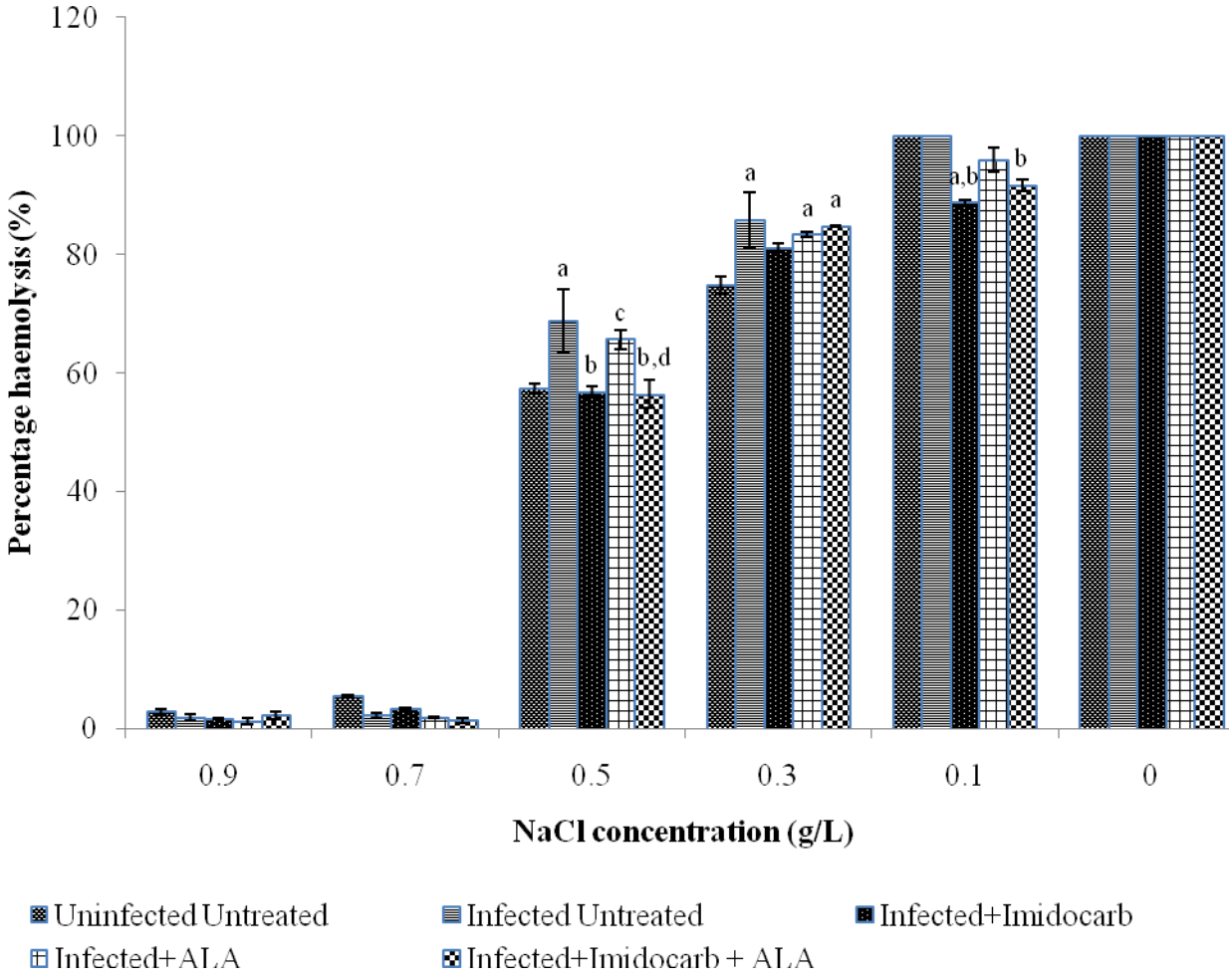


Figure 4.21: Effect of treatments on erythrocyte osmotic fragility of Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli* at day 43 PT

[a,b,c,d]P < 0.05 versus uninfected untreated, infected untreated, infected + imidocarb and infected + ALA groups, respectively

4.6.2 Effect of treatments on serum minerals

4.6.2.1 Effect of treatments on serum calcium concentration

There was no significant ($P > 0.05$) difference in the serum calcium (Ca^{2+}) concentration (Figure 4.23) within each group at base-line, days 11 PI, 8 PT, 15 PT and 43 PT, respectively. The serum Ca^{2+} concentration at day 11 PI in the uninfected untreated dogs (9.80 ± 0.09 mg/dL) was higher ($P < 0.001$) than that of the infected untreated dogs (8.17 ± 0.44 mg/dL); infected + imidocarb dogs (7.49 ± 0.21 mg/dL); infected + ALA dogs (7.55 ± 0.81 mg/dL); and infected + imidocarb + ALA dogs (8.67 ± 0.30 mg/dL; $P < 0.05$), respectively. At day 8 PT, there was a significant ($P < 0.01$) increase in the serum Ca^{2+} concentration in the uninfected untreated dogs (9.65 ± 0.26 mg/dL) compared to values recorded in the infected untreated (8.17 ± 0.44 mg/dL); infected + imidocarb (8.45 ± 0.11mg/dL; $P < 0.05$); infected + ALA (8.20 ± 0.31 mg/dL; $P < 0.001$); and infected + imidocarb + ALA (8.14 ± 0.52 mg/dL; $P < 0.05$) dogs, respectively. At day 15 PT, serum Ca^{2+} concentration increased ($P < 0.001$) in the uninfected untreated dogs (9.50 ± 0.20 mg/dL) compared to the infected untreated (7.96 ± 0.04 mg/dL); infected + imidocarb (7.74 ± 0.03 mg/dL); infected + ALA (7.79 ± 0.11 mg/dL); and infected + imidocarb + ALA (7.56 ± 0.23 mg/dL) dogs, repectively. There was no significant ($P > 0.05$) change in the serum Ca^{2+} concentration at day 43 PT between the groups.

4.6.2.2 Effect of treatments on serum phosphate concentration

Insignificant ($P > 0.05$) difference in the serum phosphate concentration (Figure 4.24) was recorded within each group at different durations. At day 11 PI, the serum phosphate concentration in the infected + imidocarb dogs (8.34 ± 1.06 mg/dL) was higher ($P < 0.05$) than the infected + imidocarb + ALA dogs (4.98 ± 0.32 mg/dL). At PT days 8, 15 and 43, there was no significant ($P < 0.05$) difference between the dogs in the different groups.

4.6.2.3 Effect of treatments on serum magnesium concentration

Magnesium (Mg^{2+}) concentration (Figure 4.25) within the groups at base-line, days 11 PI, 8 PT, 15 PT and 43 PT, did not differ respectively. Similarly, insignificant ($P > 0.05$) difference in the Mg^{2+} at days 11 PI, 8, 15 and 43 PT was recorded between the groups.

4.6.3 Effect of treatments on serum creatinine concentration

The creatinine concentration (Figure 4.26) did not differ within the groups; except in the infected + ALA dogs, in which was lower ($P < 0.05$) serum creatinine concentration was recorded at day 8 PT, (68.67 ± 12.85 mg/dL), compared to the base-line (76.71± 14.99 mg/dL) and day 43 PT (97.37 ± 11.06 mg/dL). There was no significant ($P > 0.05$) difference in the concentrations between the groups, except that of the infected + imidocarb dogs (18.75 ± 15.49 mg/dL) that was lower ($P < 0.05$) at day 11 PI, compared to the values recorded in infected + imidocarb + ALA dogs (95.62 ± 22.35 mg/dL).

4.6.4 Effect of treatments on serum blood urea nitrogen concentration

Insignificant ($P > 0.05$) difference in the blood urea nitrogen (BUN) concentration (Figure 4.27) was obtained within the infected untreated, infected + imidocarb, infected + ALA and infected + imidocarb + ALA groups. At day 11 PI, the uninfected untreated dogs (5.37 ± 0.45 mg/dL) had a lower ($P < 0.001$) BUN concentration compared to the infected untreated dogs (14.00 ± 0.96 mg/dL); infected + imidocarb dogs (10.80 ± 0.14 mg/dL); infected + ALA dogs (12.76 ± 1.77 mg/dL); and infected + imidocarb + ALA dogs (12.08 ± 1.55 mg/dL), respectively. There was no significant difference in the blood urea nitrogen concentration at days 8, 15 and 43 PT, respectively.

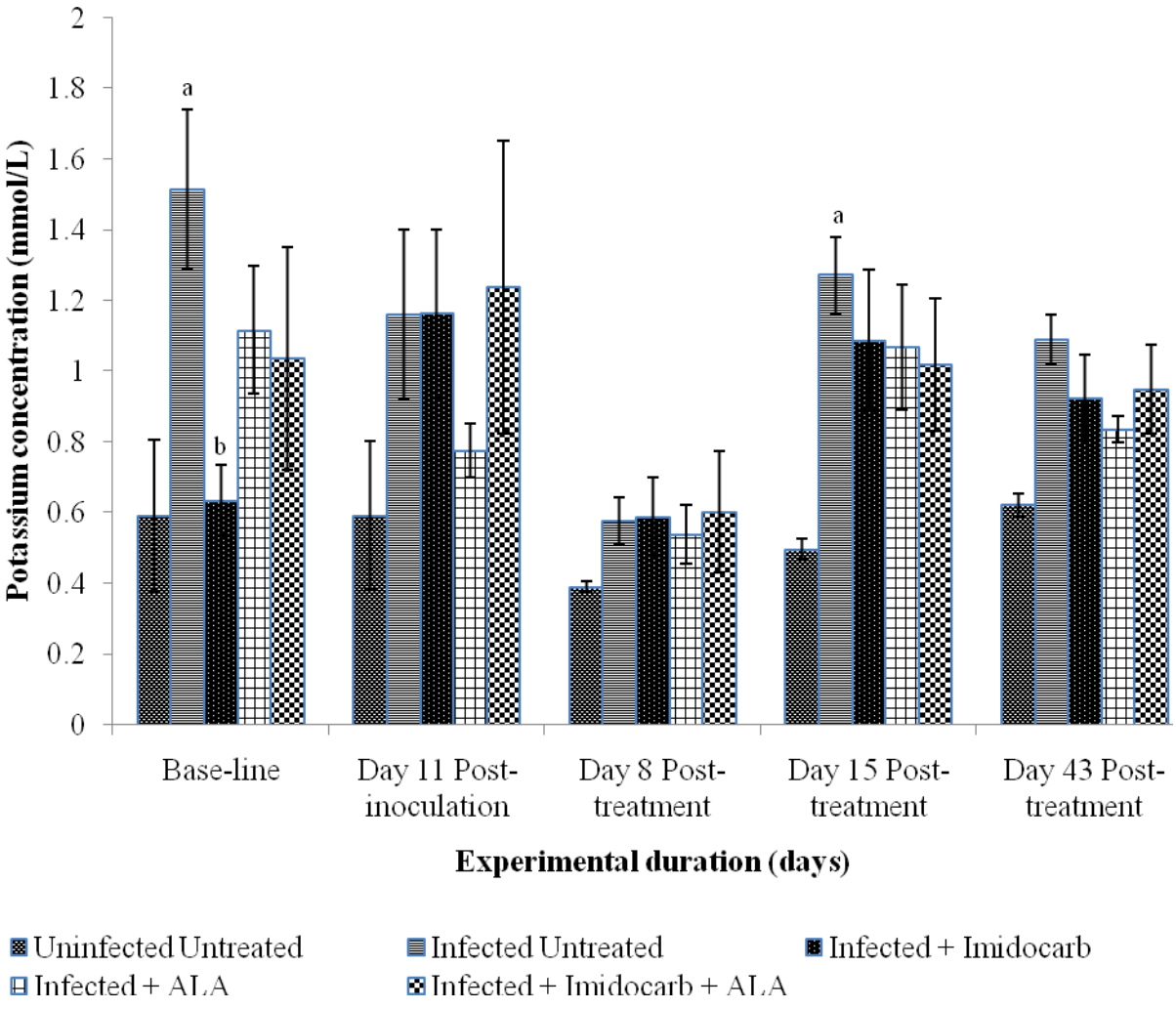


Figure 4.22: Effect of treatments on potassium in Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli*

[a,b]P < 0.05 versus uninfected untreated and infected untreated groups, respectively

Normal range: 3.6 - 5.8 mmol/L

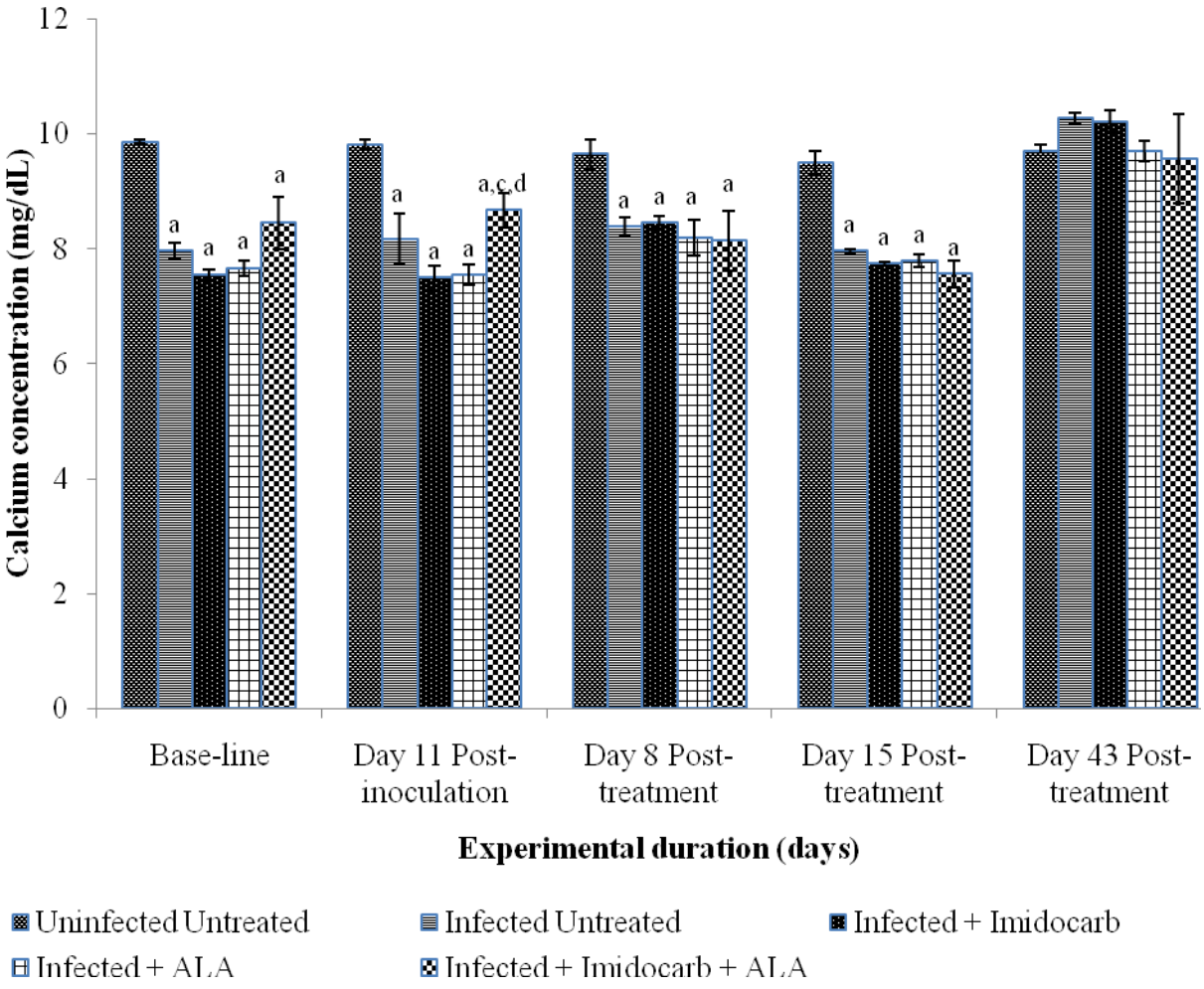


Figure 4.23: Effect of treatments on calcium in Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli*

[a,c,d]P < 0.05 versus uninfected untreated, infected + imidocarb and infected + imidocarb + ALA groups, respectively

Normal range: 7.9 - 12 mg/dL

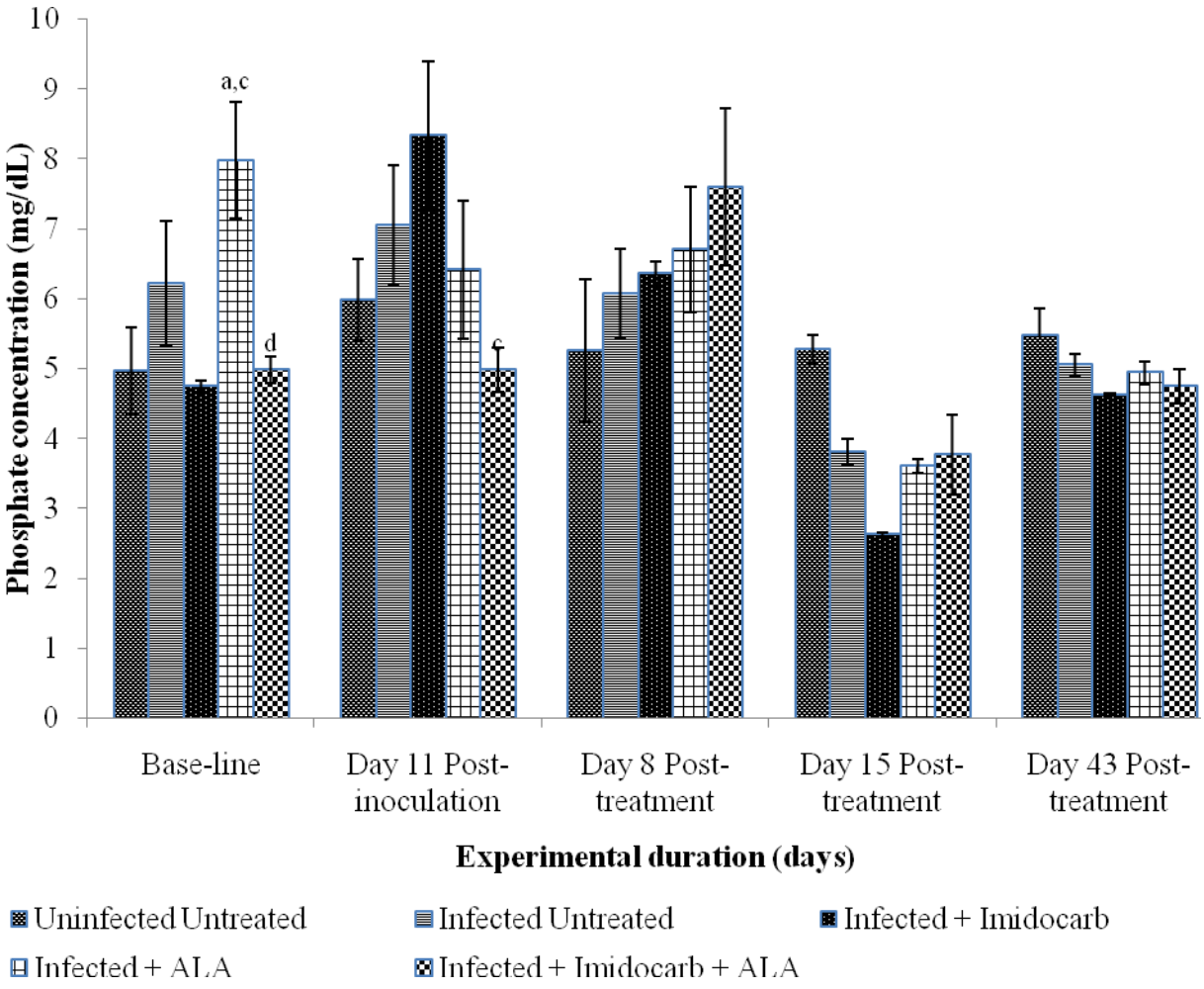


Figure 4.24: Effect of treatments on phosphate in Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli*

[a,c,d]P < 0.05 versus uninfected untreated, infected + imidocarb and infected + imidocarb + ALA groups, respectively

Normal range: 3.3 - 5.5 mg/dL

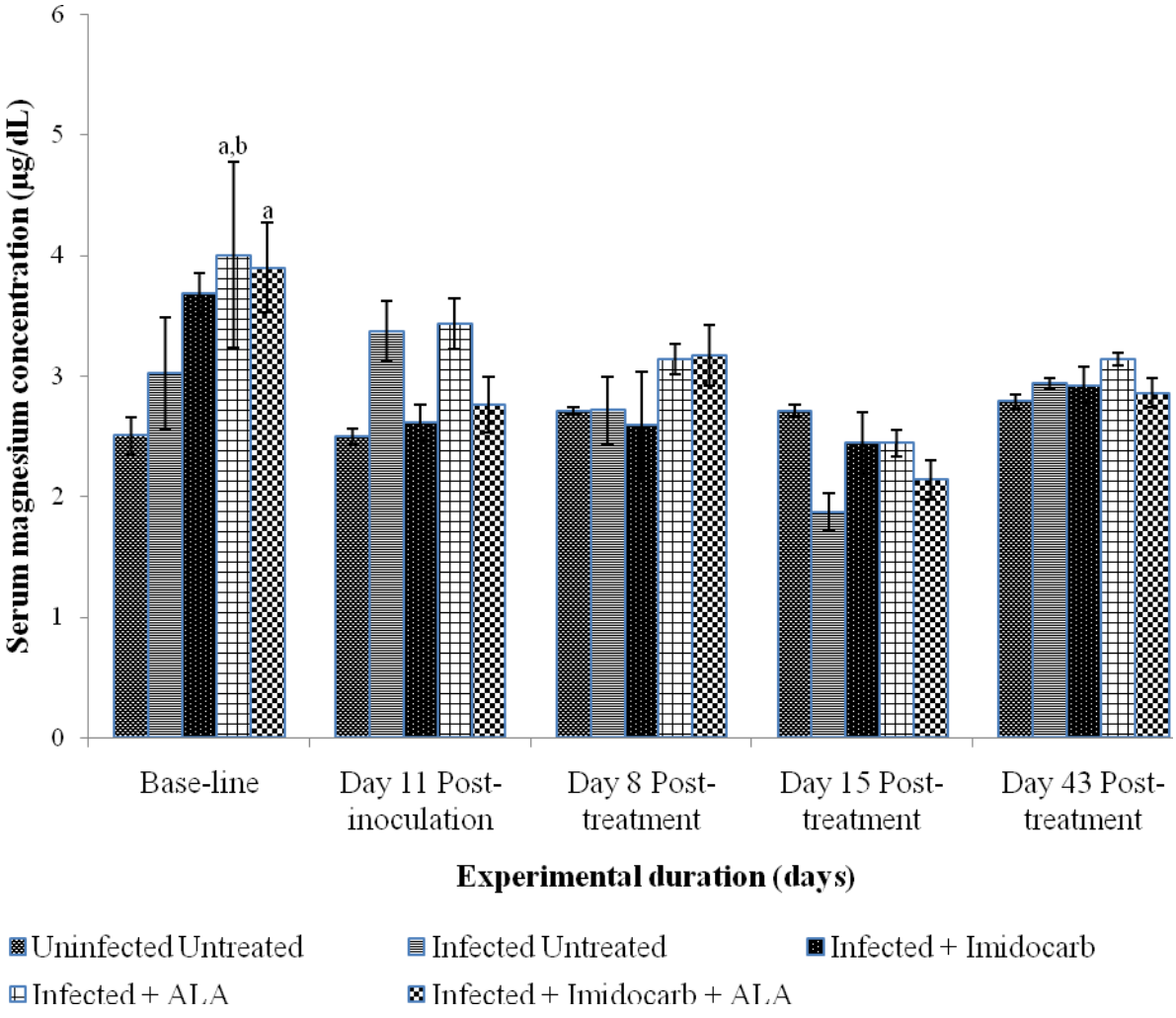


Figure 4.25: Effect of treatments on serum magnesium concentration in Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli*

[a,b]P < 0.05 versus uninfected untreated and infected untreated groups, respectively

Normal range: 1.89 - 2.50 µg/dL

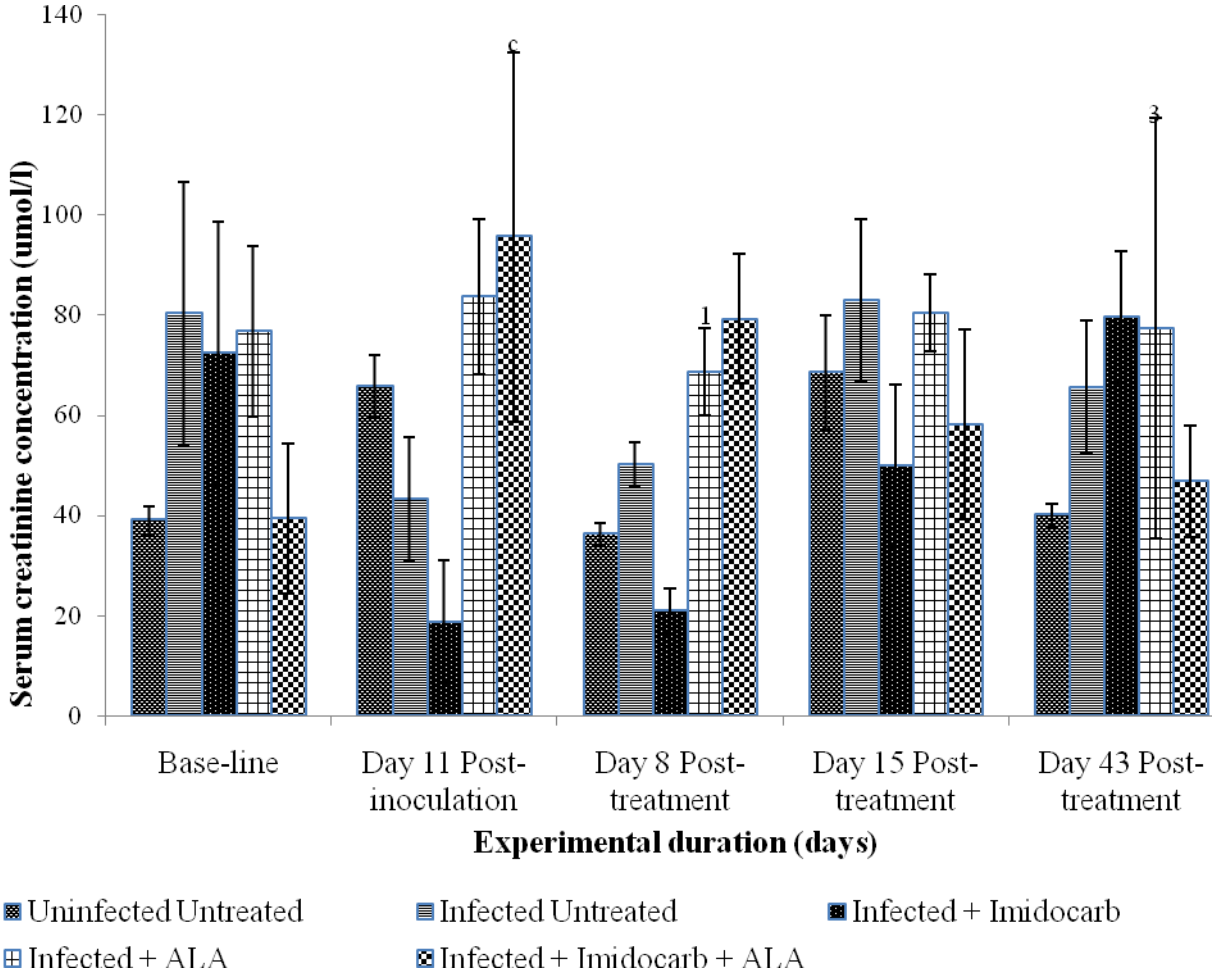


Figure 4.26: Effect of treatments on creatinine concentration in Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli*

[1,3]P < 0.05 versus base-line and day 8 PT, respectively
[c]P < 0.05 versus infected + imidocarb group

Normal range: 44 - 133 mmol/L

4.6.5 Effect of treatments on serum bilirubin concentration

The serum bilirubin concentration (Figure 4.28) was not significant ($P > 0.05$) at base-line, days 11 PI, 8 PT, 15 PT and 43 PT respectively within the groups. At day 11 PI, lower ($P < 0.001$) serum bilirubin concentration was recorded in the uninfected untreated dogs (2.13 ± 0.91 µmol/L) compared to the values obtained in the infected untreated (26.49 ± 5.64 µmol/L); infected + imidocarb (34.73 ± 7.66 µmol/L); infected + ALA (31.78 ± 5.23 µmol/L); and infected + imidocarb + ALA (29.15 ± 0.54 µmol/L) dogs. At day 8 PT, there was no significant difference in the serum bilirubin concentration between the groups. At day 15 PT, the uninfected untreated dogs (2.69 ± 1.02 µmol/L) had a lower ($P < 0.05$) serum bilirubin concentration, compared to the infected untreated dogs (14.45 ± 3.62 µmol/L). At day 43 PT, the difference in the serum bilirubin concentration between the groups was insignificant.

4.6.6 Effect of treatments on serum creatine kinase concentration

Creatine kinase (CK) activities were not significantly different (Figure 4.29) within the groups at base-line, days 11 PI, 8 PT, 15 PT and 43 PT, respectively. At day 11 PI, the uninfected untreated dogs (229.72 ± 39.92 µ/L) had a significantly ($P < 0.05$) higher serum CK activities compared to the infected untreated (116.22 ± 34.52 µ/L) and infected + imidocarb (62.42 ± 15.87 µ/L) dogs. The activity of CK in infected + imidocarb + ALA dogs (216.37 ± 51.54 µ/L) was higher than that of infected + imidocarb dogs (62.42 ± 15.87 µ/L). At days 8 and 15 PT, there was no significant ($P > 0.05$) difference in the CK activities between the groups. At day 43 PT, the serum CK activity in the uninfected untreated dogs (331.26 ± 36.65 µ/L) was significantly ($P < 0.001$) higher, compared to the corresponding activities recorded in the infected untreated dogs (98.64 ± 17.70 µ/L);

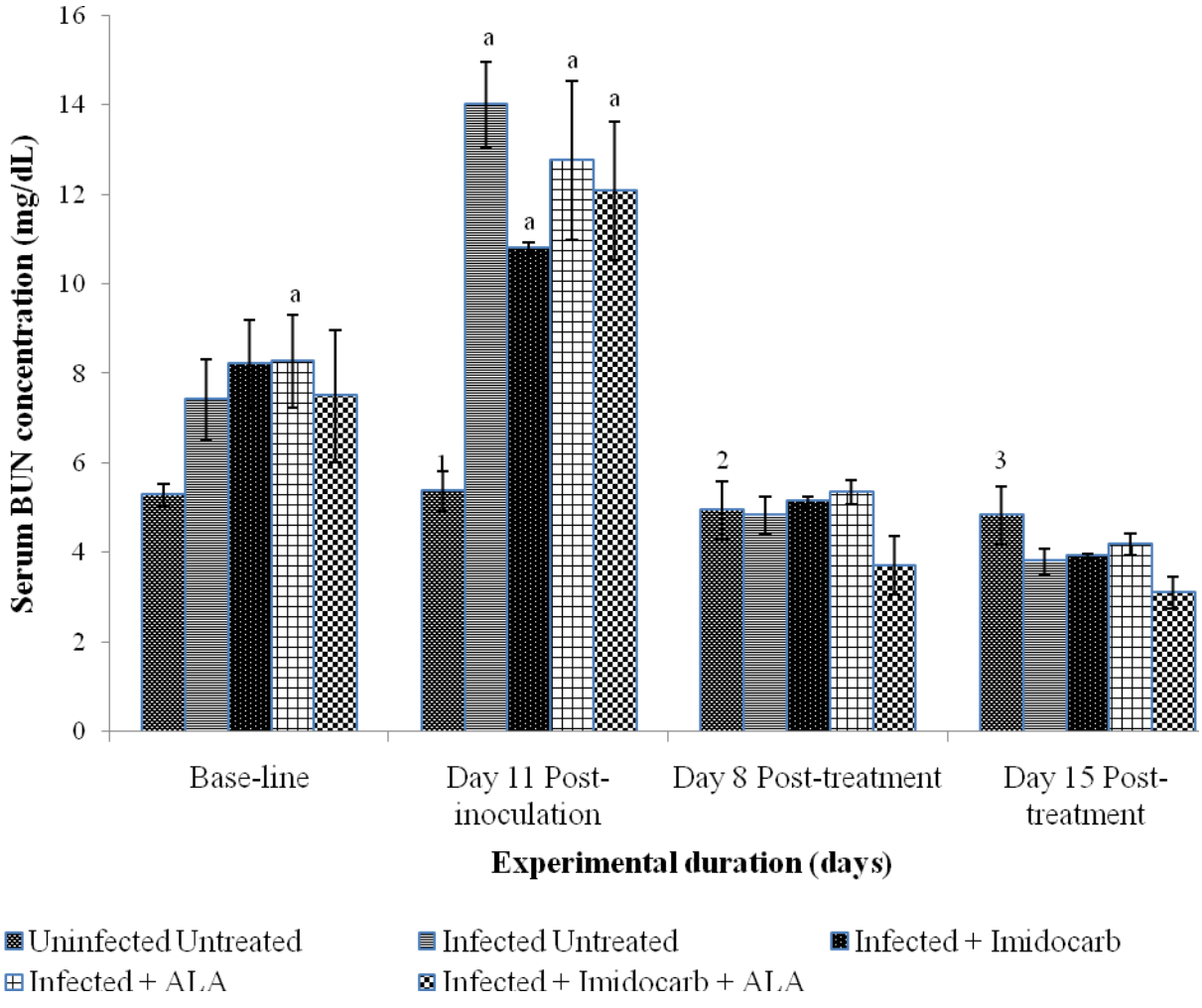


Figure 4.27: Effect of treatments on blood urea nitrogen concentration in Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli*

[1,2,3]P < 0.05 versus base-line, days 11 PI and 8 PT, respectively
[a]P < 0.05 versus uninfected untreated group

Normal range: 7 - 32 mg/dL

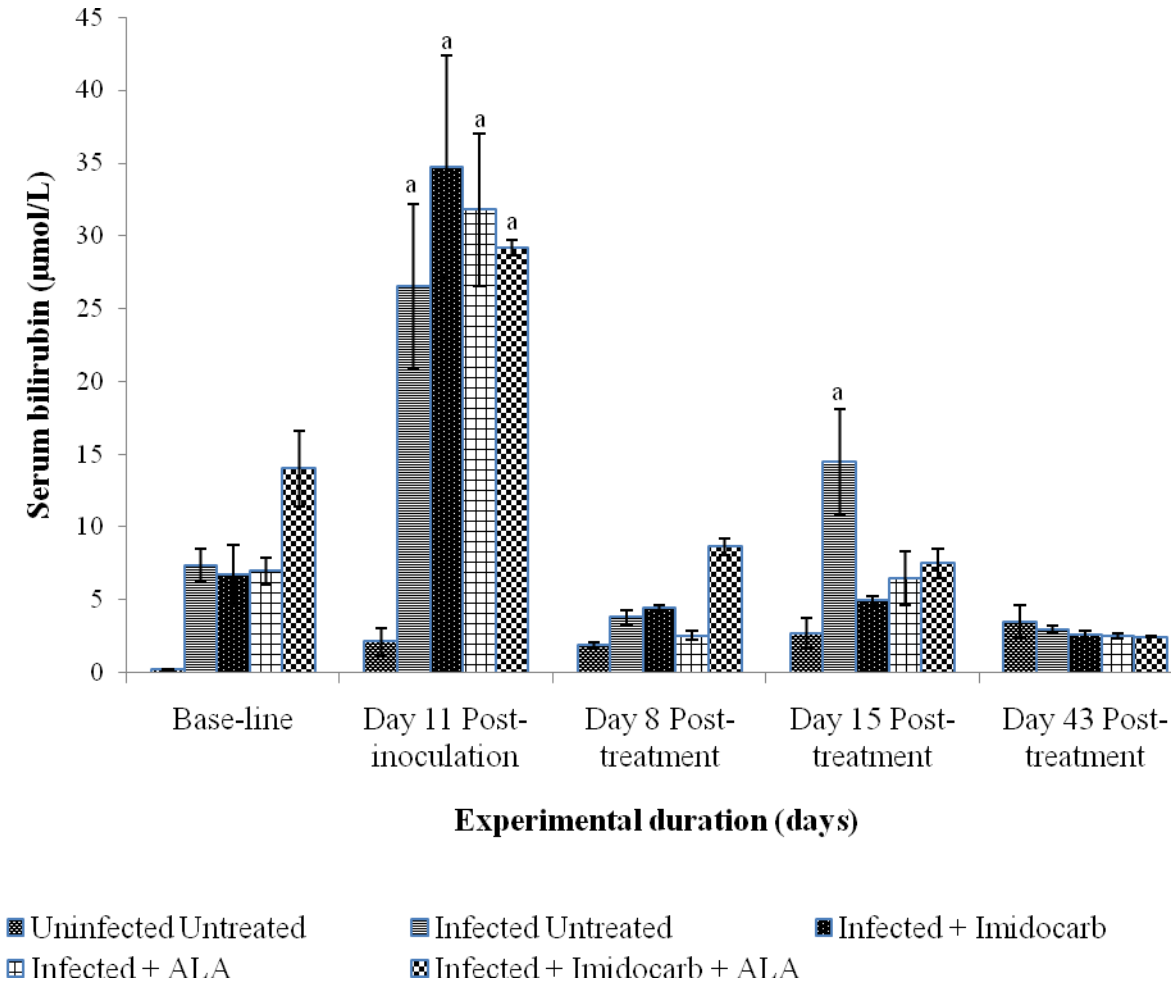


Figure 4.28: Effect of treatments on serum bilirubin concentration in Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli*

[a]P < 0.05 versus uninfected untreated group

Normal range: 0 - 1.71 µmol/L

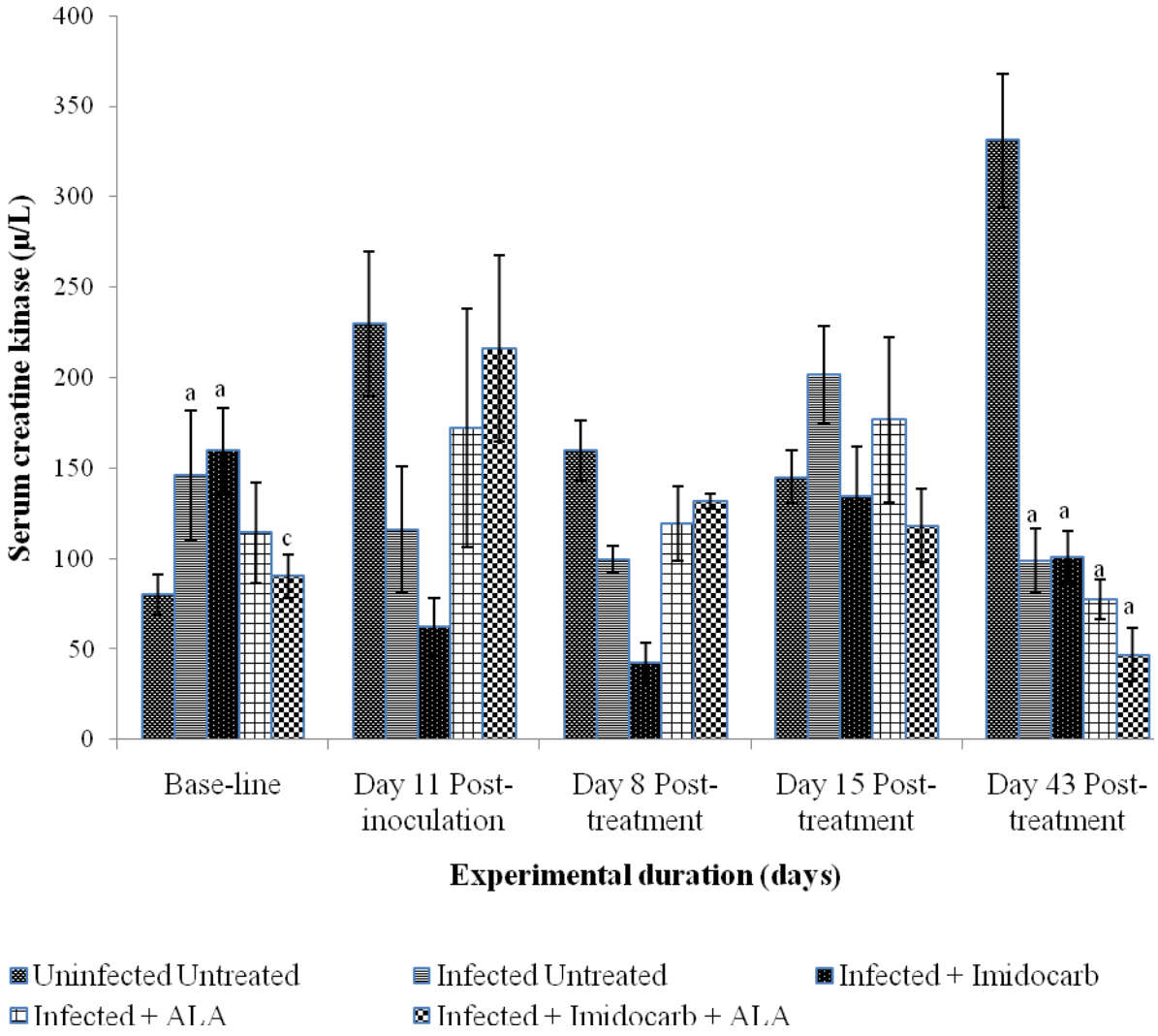


Figure 4.29: Effect of treatments on serum creatine kinase concentration in Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli*

[a,c]P < 0.05 versus uninfected untreated and infected + imidocarb groups, respectively

Normal range: 40 - 254 µ/L

infected + imidocarb dogs (100.75 ± 14.76 µ/L); infected + ALA dogs (77.37 ± 11.06 µ/L); and infected + imidocarb + ALA (46.76 ± 14.87 µ/L).

4.6.7 Effect of treatments on serum proteins

4.6.7.1 Effect of treatments on serum total protein

The difference in the total protein concentration (TP) (Figure 4.30) within the groups at base-line, days 11 PI, PT 8, 15 and 43, respectively was insignificant. At day 11 PI, the uninfected untreated dogs (5.28 ± 0.33 g/dL) had significantly ($P < 0.001$) lower TP concentration compared to those of the infected untreated dogs (17.00 ± 2.15 g/dL), infected + imidocarb dogs (17.81 ± 2.49 g/dL), infected + ALA dogs (17.69 ± 1.29 g/dL), and infected + imidocarb + ALA dogs (16.27 ± 1.93 g/dL). At PT days 8 and 15, there was no significant ($P > 0.05$) difference between the groups. At day 43 PT, the uninfected untreated dogs (5.31 ± 0.46 g/dL) had lower ($P < 0.001$) TP concentration, compared to the values obtained in dogs in the infected untreated group (12.22 ± 0.47 g/dL), infected + imidocarb group (12.05 ± 0.39 g/dL), infected + ALA group (9.68 ± 1.28 g/dL; $P < 0.05$), and infected + imidocarb + ALA group (10.61 ± 0.62 g/dL; $P < 0.05$).

4.6.7.2 Effect of treatments on serum albumin

The albumin concentration (Figure 4.31) was insignificant ($P > 0.05$) within the groups at base-line, days 11 PI, 8 PT, 15 PT and 43 PT, respectively. At day 11 PI, there was a significant ($P < 0.01$) increase in the serum albumin concentration in the uninfected untreated dogs (4.15 ± 0.36 g/dL), compared to the infected untreated dogs (2.20 ± 0.47 g/dL) and the infected + imidocarb + ALA dogs (2.23 ± 0.49 g/dL). At day 8 PT, serum albumin concentration was higher ($P < 0.001$) in the uninfected untreated dogs (4.72 ± 0.92 g/dL), compared to the infected untreated dogs (2.21 ± 0.16 g/dL), infected + imidocarb

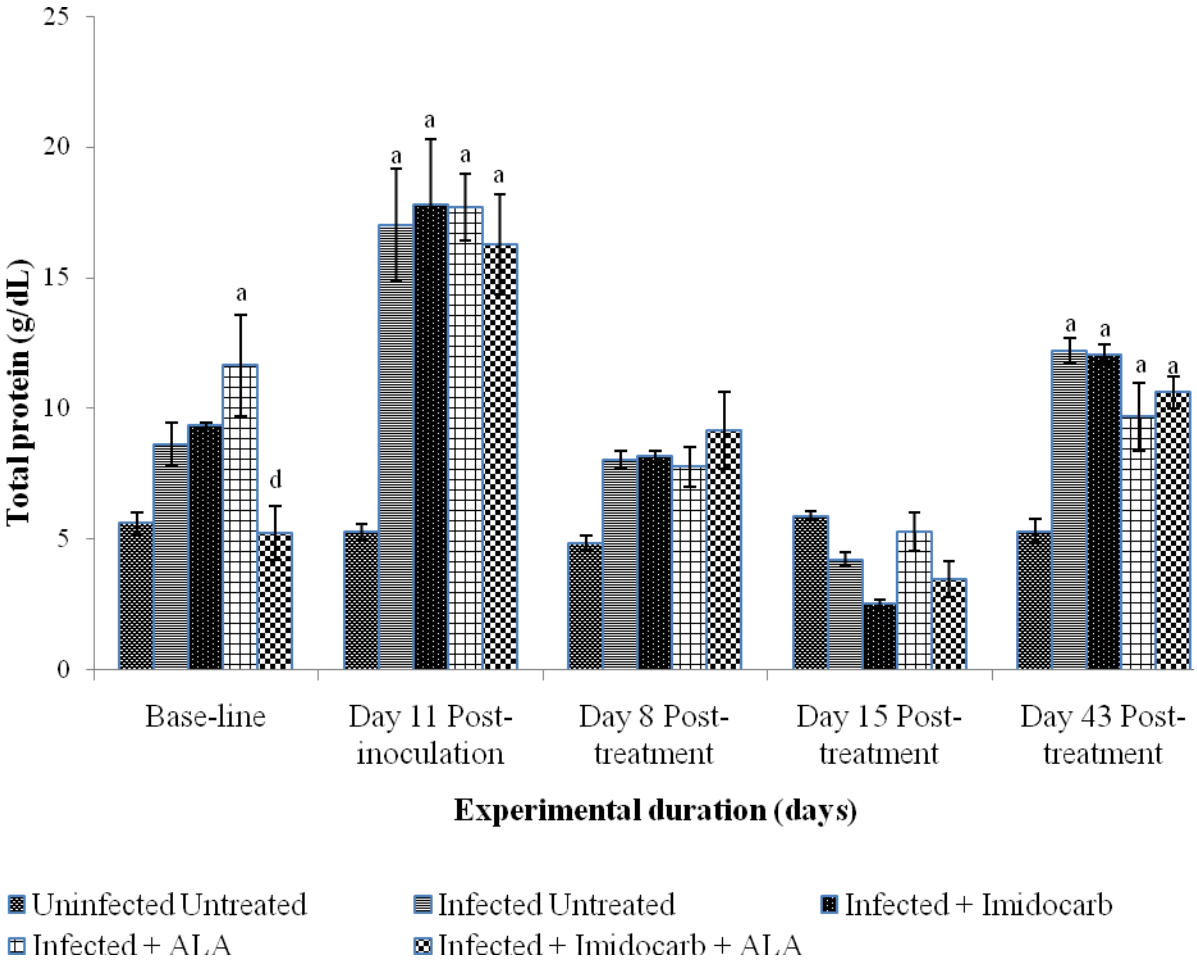


Figure 4.30: Effect of treatments on total protein in Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli*

[a,d]P < 0.05 versus uninfected untreated and infected + ALA groups, respectively

Normal range: 5.4 - 7.5 g/dL

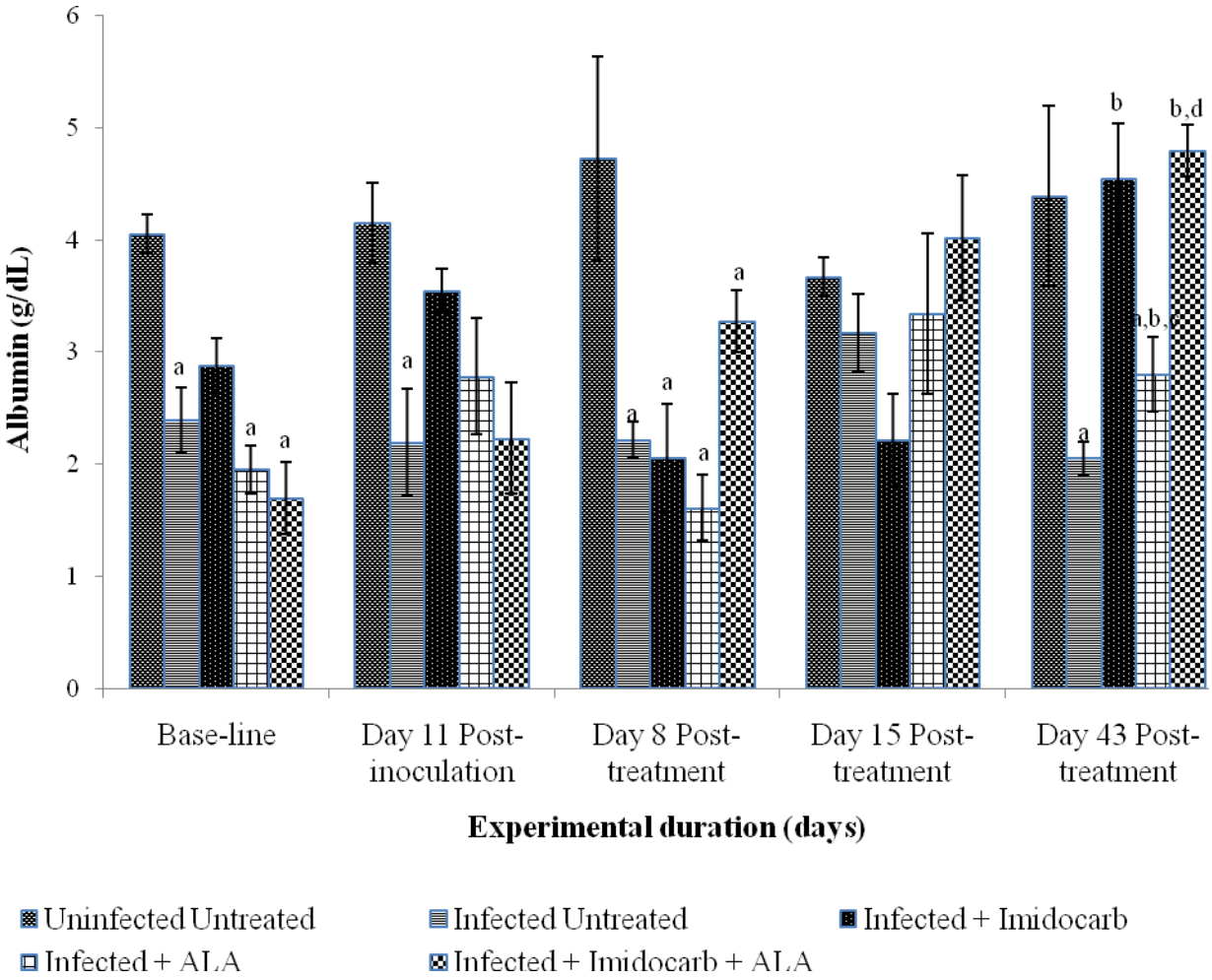


Figure 4.31: Effect of treatments on albumin in Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli*

[a,b,c,d]P < 0.05 versus uninfected untreated, infected untreated, infected + imidocarb and infected + imidocarb +ALA groups, respectively

Normal range: 3.4 - 4.3 g/dL

dogs (2.05 ± 0.49 g/dL), and infected + ALA dogs (1.61 ± 0.29 g/dL). At day 15 PT, no significant ($P > 0.05$) difference in the serum albumin concentrations was obtained between the groups. At day 43 PT, the serum albumin concentration in the uninfected untreated dogs (4.39 ± 0.81 g/dL) was higher ($P < 0.001$) than the infected untreated dogs (2.50 ± 0.15 g/dL) or infected + ALA dogs (2.80 ± 0.33 g/dL). Serum albumin concentration was lower ($P < 0.001$) in the infected untreated dogs (2.05 ± 0.15 g/dL), compared to the concentrations recorded in the infected + imidocarb dogs (4.55 ± 0.50 g/dL), infected + ALA dogs (2.80 ± 0.33 g/dL; $P < 0.05$) and infected + imidocarb + ALA dogs (4.80 ± 0.23 g/dL). Significant decrease was also recorded in the infected + ALA dogs compared to the infected + imidocarb + ALA dogs.

4.6.7.3 Effect of treatments on serum globulin

There was no significant ($P > 0.05$) difference in the serum globulin concentrations (Figure 4.32) within the groups at base-line, days 11 PI, 8 PT, 15 PT and 43 PT, respectively. At base-line, the serum globulin concentration in the infected + ALA dogs (9.69 ± 2.00 g/dL) was higher ($P < 0.001$) than those of the uninfected untreated dogs (1.56 ± 0.38 g/dL); infected untreated dogs (5.18 ± 1.20 g/dL); and infected + imidocarb + ALA dogs (3.53 ± 1.22 g/dL). At day 11 PI, uninfected untreated dogs (1.20 ± 0.57 g/dL) had a lower ($P < 0.001$) serum globulin concentration compared to the infected untreated dogs (14.80 ± 2.10 g/dL), infected + imidocarb dogs (14.26 ± 2.30 g/dL), infected + ALA dogs (14.90 ± 1.27 g/dL), and infected + imidocarb + ALA dogs (14.04 ± 2.41 g/dL). At day 8 PT, the globulin concentration in the uninfected untreated dogs (0.13 ± 0.81 g/dL) was lower ($P < 0.01$) than in any of the following groups; infected untreated dogs (5.83 ± 0.45 g/dL), infected + imidocarb dogs (6.13 ± 0.48 g/dL), infected + ALA dogs (6.17 ± 0.63 g/dL), and infected + imidocarb + ALA dogs (5.87 ± 1.22 g/dL). There was no significant ($P > 0.05$) difference

in the concentration at day 15 PT between the groups. At day 43 PT, the uninfected untreated dogs (1.88 ± 0.74 g/dL) a lower ($P < 0.001$) globulin concentration was recorded compared to the concentrations obtained in the infected untreated dogs (10.17 ± 0.51 g/dL), infected + imidocarb dogs (7.51 ± 0.30 g/dL). There was significant ($P < 0.01$) increase in the globulin concentration infected untreated dogs (10.17 ± 0.51 g/dL), compared to the value recorded in the infected + ALA dogs (6.03 ± 1.48 g/dL).

4.6.7.4 Effect of treatments on serum albumin/globulin ration concentration

The difference in the albumin/globulin (A/G) ratio (Figure 4.33) within and between the groups at base-line, days 11 PI, 8 PT, 15 PT and 43 PT did not differ significantly.

4.7 Effect of Treatments on Liver Enzymes Activities

4.7.1 Effect of treatments on alanine transaminase activity

The difference in the alanine transaminase (ALT) activities (Figure 4.34) within the groups at base-line, days 11 PI, PT 8, 15 and 43, respectively was not significant. At base-line, there was no significant ($P > 0.05$) difference in the ALT activities between the groups. At day 11 PI, the uninfected untreated dogs (16.00 ± 0.71 U/L) had a lower ($P < 0.05$) ALT activity compared to the infected untreated dogs (22.67 ± 2.88 U/L). At PT days 8, 15 and 43, there was no significant ($P > 0.05$) difference in the ALT activity between the groups.

4.7.2 Effect of treatments on aspartate transaminase activity

There was no significant ($P > 0.05$) difference in the aspartate transaminase (AST) activities (Figure 4.35) within the groups at base-line, days 11 PI, 8 PT, 15 PT and 43 PT, respectively. Insignificant ($P > 0.05$) difference in the AST activities was recorded between

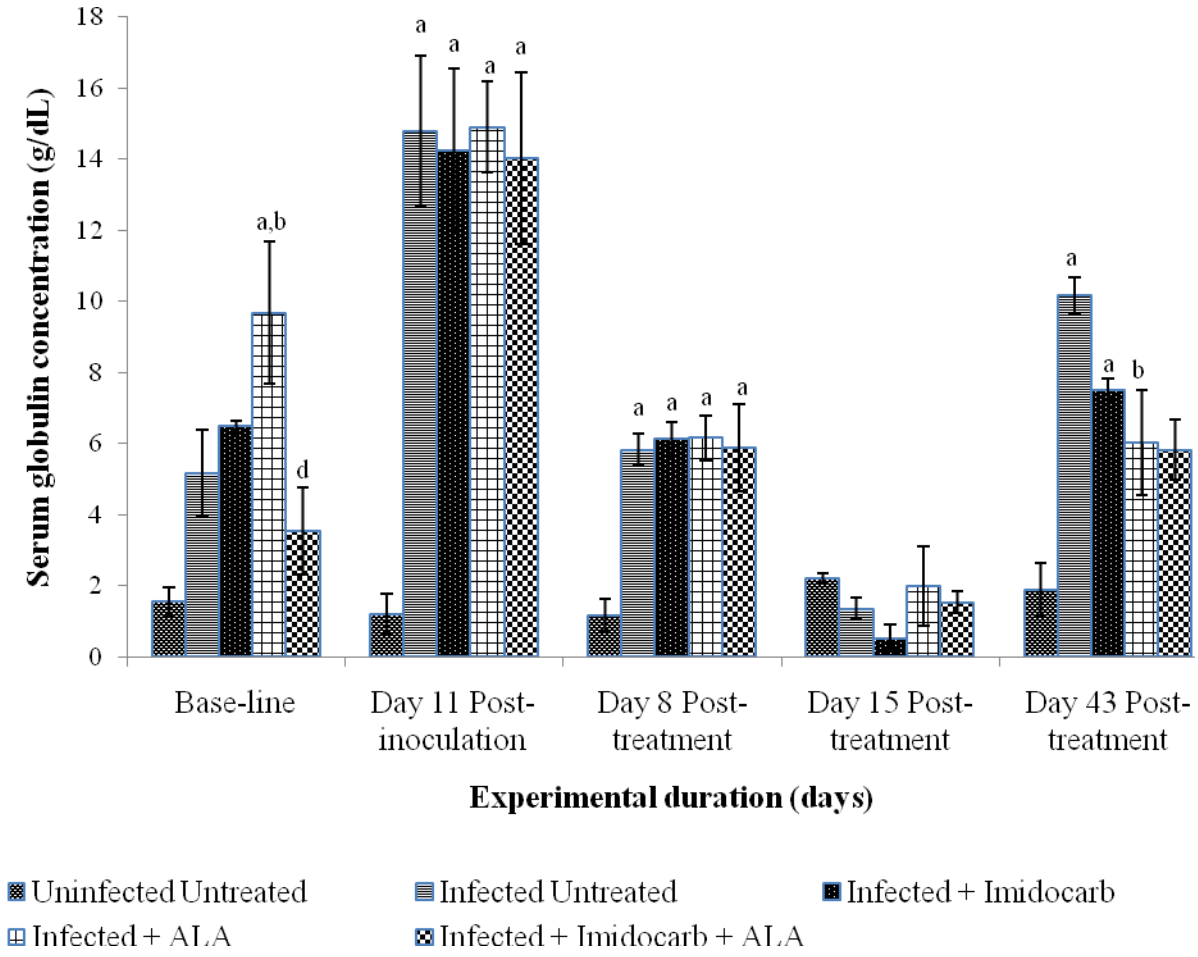


Figure 4.32: Effect of treatments on globulin in Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli*

[a,b,d]P < 0.05 versus uninfected untreated, infected untreated and infected + ALA groups, respectively

Normal range: 2.1 - 4.1 g/dL

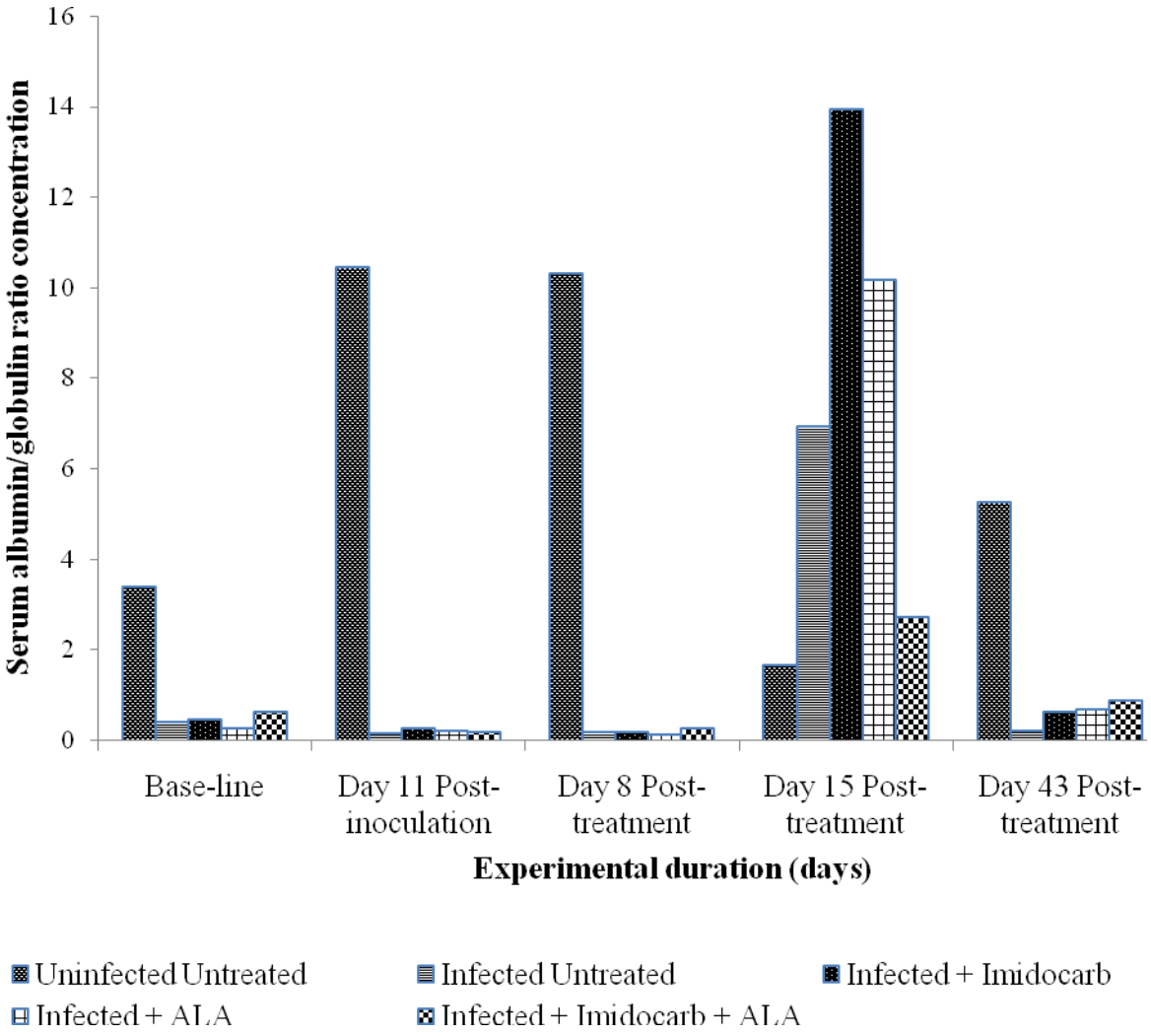


Figure 4.33: Effect of treatments on albumin/globulin ratio in Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli*

Normal range: 0.7 - 1.0

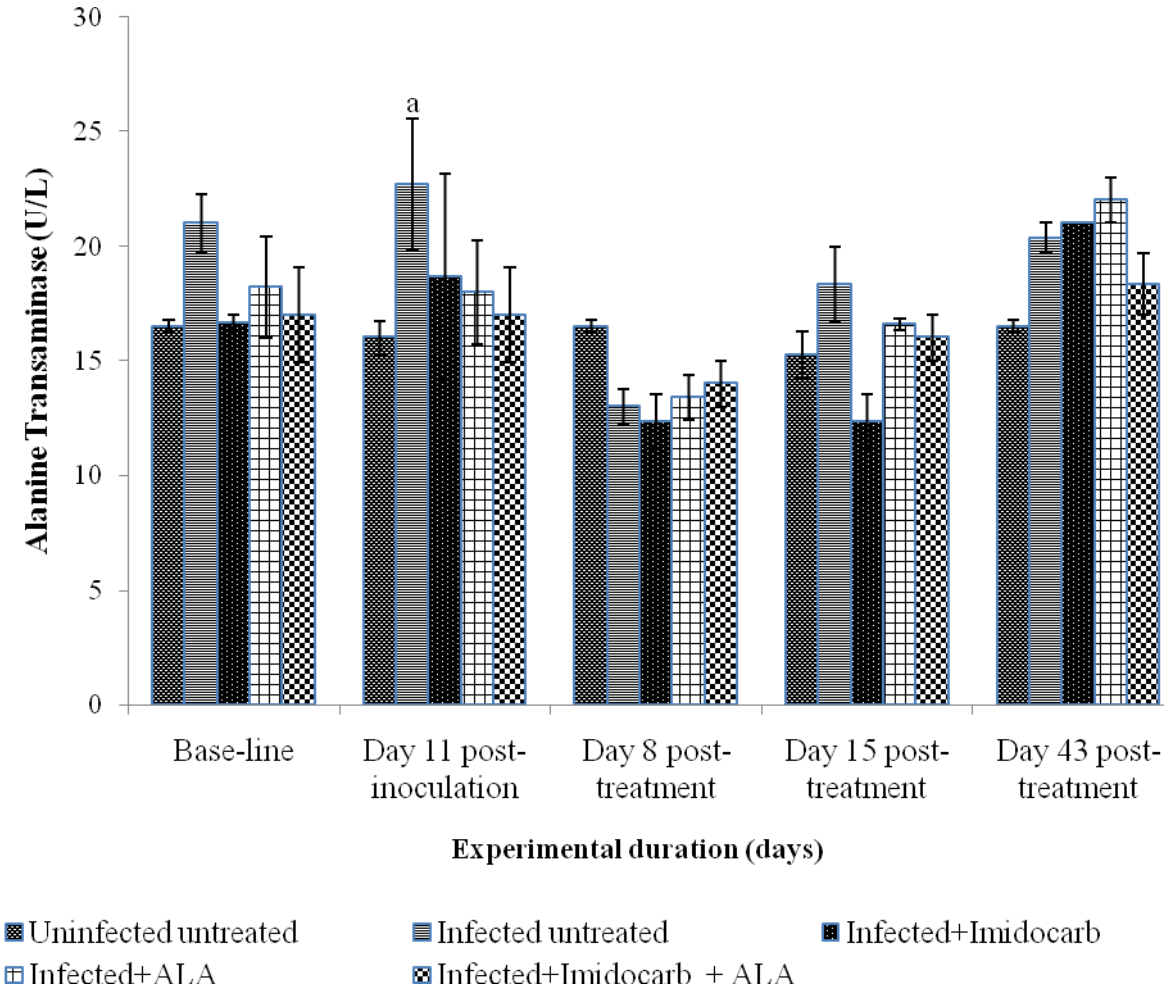


Figure 4.34: Effect of treatments on alanine transaminase activity in Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli*

[a]P < 0.05 versus uninfected untreated group

Normal range: 8.2 - 57 U/L

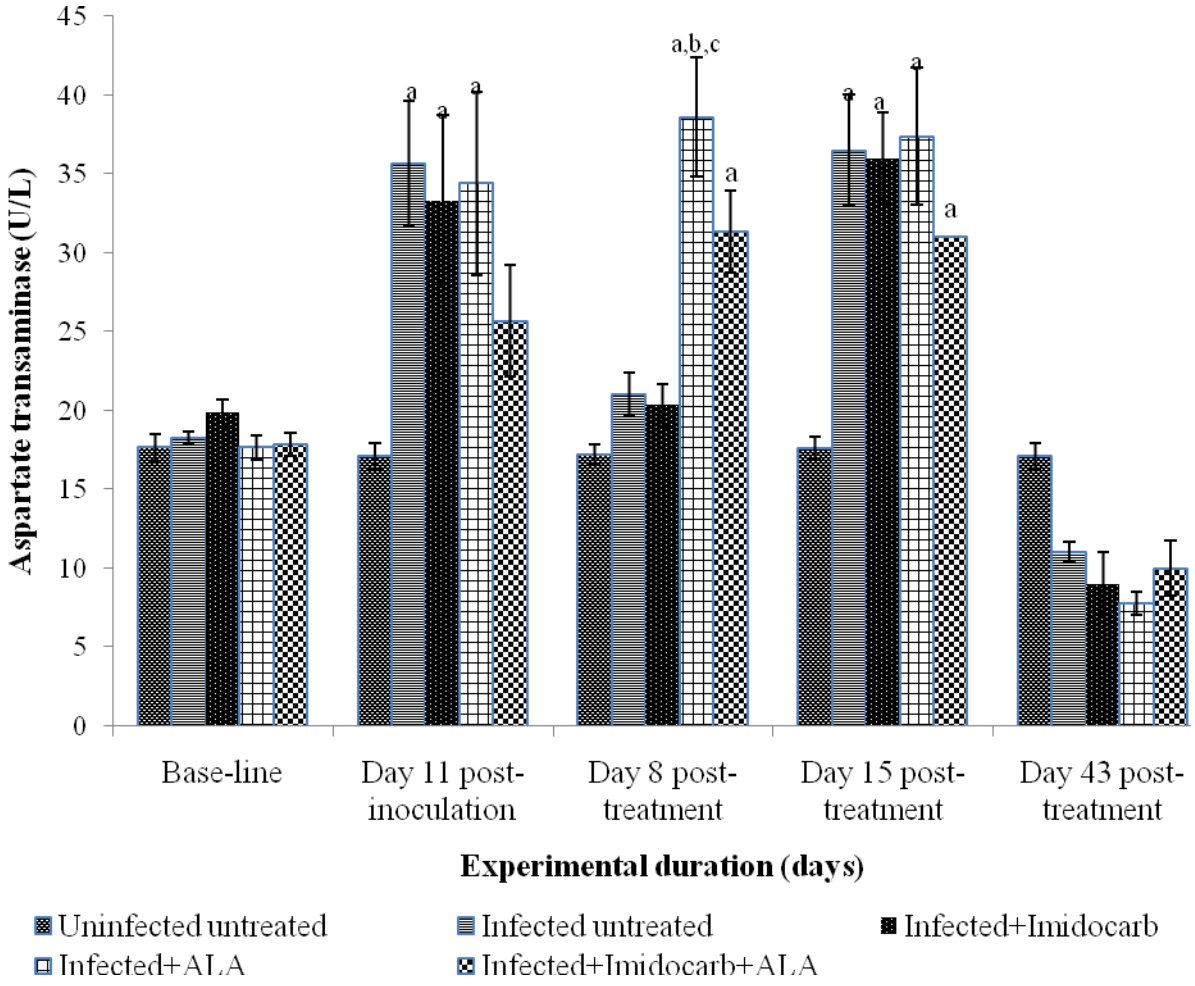


Figure 4.35: Effect of treatments on aspartate transaminase activity in Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli*

[a,b,c]P < 0.05 versus uninfected untreated, infected untreated and infected + imidocarb groups, respectively

Normal range: 16 - 43 U/L

the groups at base-line. At day 11 PI, the AST activity in the uninfected untreated dogs (17.11 ± 0.83 U/L) was significantly ($P < 0.001$) lower than that recorded in the infected untreated dogs (35.67 ± 3.95 U/L); infected + imidocarb dogs (33.33 ± 5.36 U/L; $P < 0.01$) and infected + ALA dogs (34.40 ± 5.83 U/L; $P < 0.001$), respectively. At day 8 PT, the uninfected untreated dogs (17.22 ± 0.63 U/L) had a lower ($P < 0.001$) AST activity compared to the infected + ALA dogs (38.60 ± 3.80 U/L) and infected + imidocarb + ALA dogs (31.33 ± 2.60 U/L; $P < 0.05$).

The AST activity in the infected + ALA dogs (38.60 ± 0.98 U/L) was higher ($P < 0.001$), compared to the infected untreated dogs (21.00 ± 1.37 U/L) and the infected + imidocarb dogs (20.33 ± 1.33 U/L), respectively. At day 15 PT, the uninfected untreated dogs (17.61 ± 0.76 U/L) had a lower ($P < 0.001$) AST activities than the infected untreated dogs (36.50 ± 3.52 U/L); infected + imidocarb dogs (36.00 ± 2.89 U/L); infected + ALA dogs (37.40 ± 0.24 U/L); and infected + imidocarb + ALA dogs (31.00 ± 0.00 U/L; $P < 0.05$), respectively. At day 43 PT, there was no significant ($P > 0.05$) difference in the AST activities between the groups.

4.7.3 Effect of treatments on alkaline phosphatase activity

The alkaline phosphatase (ALP) activity (Figure 4.36) within the groups at base-line, days 11 PI, 8 PT, 15 PT and 43 PT, respectively were not different. There was no significant ($P > 0.05$) change at base-line between the groups. At day 11 PI, ALP activity in the uninfected untreated dogs (54.16 ± 13.29 U/L) was significantly ($P < 0.01$) lower than that of the infected + imidocarb + ALA dogs (182.77 ± 11.77 U/L). At day 8 PT, the ALP activity in the uninfected untreated dogs (54.88 ± 22.74 U/L) was lower ($P < 0.01$) than that recorded in the infected + ALA dogs (184.41 ± 7.23 U/L).

At day 15 PT, there was no significant ($P > 0.05$) difference in the ALP activities between the groups. At day 43 PT, the uninfected untreated dogs (31.62 ± 6.58 U/L) had a lower ($P < 0.05$) ALP activities compared to the infected untreated dogs (136.08 ± 18.07 U/L); infected + ALA dogs (247.96 ± 49.97 U/L; $P < 0.001$); and infected + imidocarb + ALA dogs (198.08 ± 16.58 U/L; $P < 0.001$) dogs, respectively. The infected + ALA dogs (247.96 ± 49.97 U/L) had higher ($P < 0.01$) ALP activities than the infected untreated dogs (136.08 ± 18.07 U/L) and infected + imidocarb dogs (114.66 ± 30.83 U/L), respectively.

4.7.4 Effect of treatments on gamma-glutamyl transferase activity

The gamma-glutamyltransferase (GGT) activities (Figure 4.37) within the groups at base-line, days 11 PI, 8 PT, 15 PT and 43 PT, respectively were insignificant. There was no significant ($P > 0.05$) difference at base-line between the groups. At day 11 PI, the GGT activity in the uninfected untreated dogs (2.92 ± 1.22 U/L) was significantly ($P < 0.001$) lower than the activity obtained in the infected untreated dogs (17.90 ± 4.65 U/L) and the infected + imidocarb dogs (27.79 ± 6.78 U/L), respectively. The GGT activity in the infected untreated dogs (17.90 ± 4.65 U/L) was lower ($P < 0.05$) than in the infected + imidocarb dogs (27.79 ± 6.78 U/L). The GGT activity in the infected untreated dogs (17.90 ± 4.65 U/L) was higher ($P < 0.001$) compared to the infected + ALA dogs (3.83 ± 1.57 U/L) and infected + imidocarb + ALA dogs (5.45 ± 0.52 U/L; $P < 0.01$), respectively. There was a significant ($P < 0.001$) increase in the GGT activity in the infected + imidocarb dogs (27.79 ± 6.78 U/L) compared to the infected + ALA dogs (3.83 ± 1.57 U/L) and infected + imidocarb + ALA dogs (5.45 ± 0.52 U/L), respectively. There was no significant ($P > 0.05$) difference in the GGT activity between the groups at days 8, 15 and 43 PT, respectively.

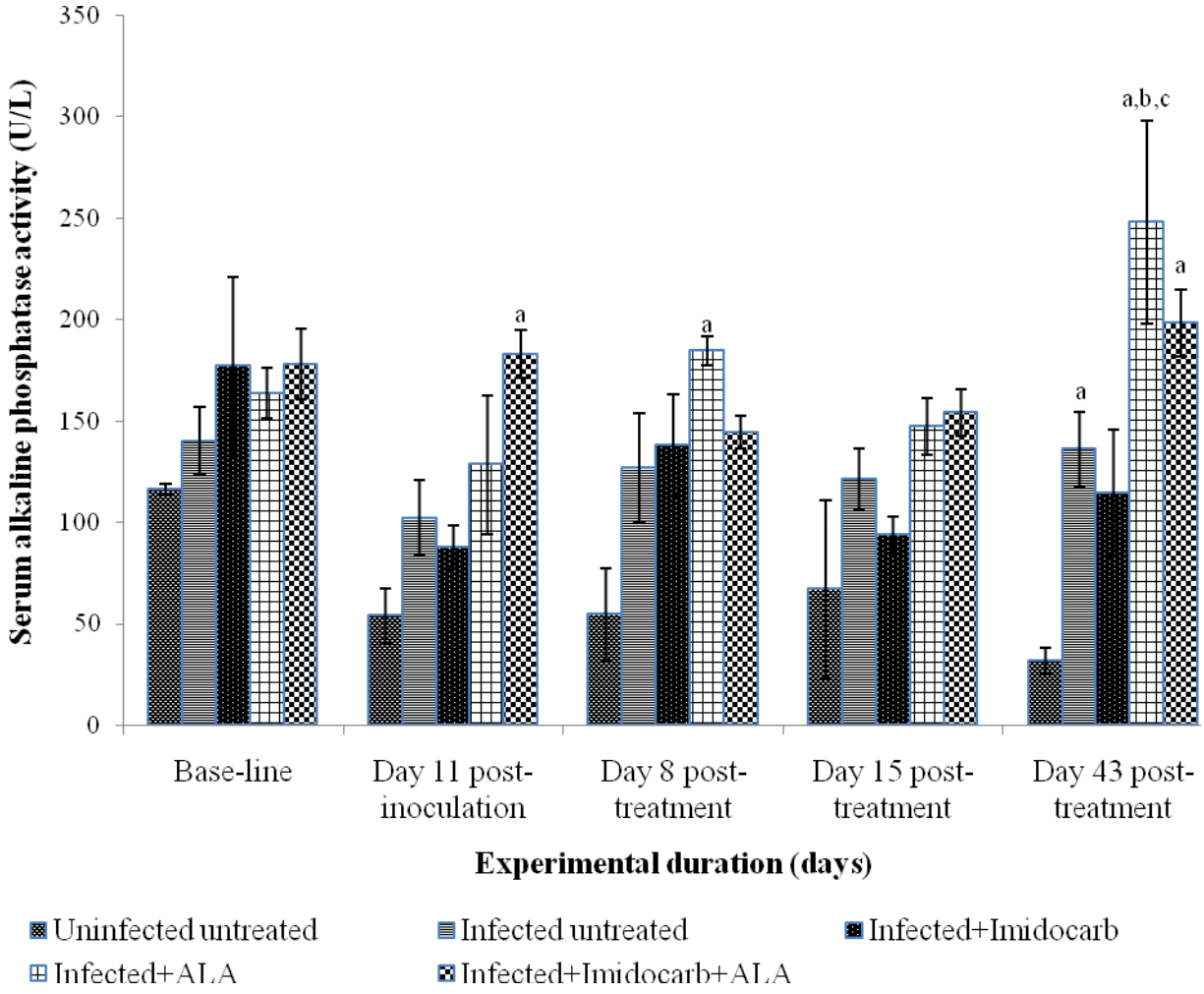


Figure 4.36: Effect of treatments on alkaline phosphatase activity in Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli*

[a,b,c]P < 0.05 versus uninfected untreated, infected untreated and infected + imidocarb groups, respectively

Normal range: 10 - 75 U/L

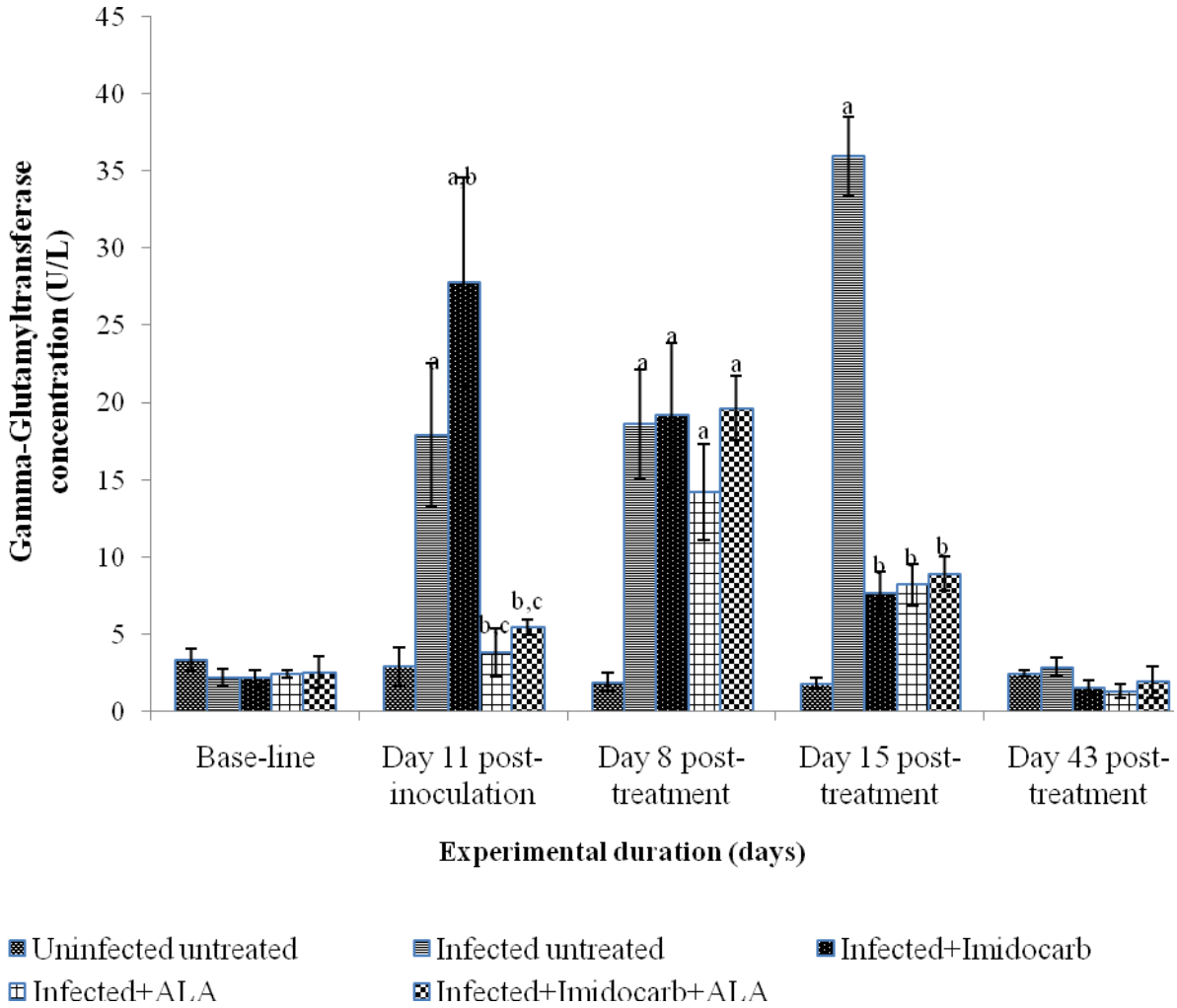


Figure 4.37: Effect of treatments on gamma-glutamyltransferase activity in Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli*

[a,b,c]P < 0.05 versus uninfected untreated, infected untreated and infected + imidocarb groups, respectively

Normal range: 1 - 5 U/L

4.8 Effect of treatments on Serum Trace Elements

4.8.1 Effect of treatments on serum copper concentration

The difference in copper (Cu^{2+}) concentrations (Figure 4.38) within the groups at base-line, days 11 PI, 8 PT, 15 PT and 43 PT, respectively was insignificant. At base-line and days 11 PI, there was no significant ($P > 0.05$) difference in the Cu^{2+} concentration between the groups. At day 8 PT, the Cu^{2+} concentration in the uninfected untreated dogs (40.67 ± 4.36 µmol/L) was significantly ($P < 0.01$) higher than the concentrations in the infected untreated dogs (24.53 ± 4.17 µmol/L); infected + imidocarb dogs (22.94 ± 0.64 µmol/L; $P < 0.05$) and infected + ALA dogs (23.87 ± 1.50 µmol/L), respectively. At day 15 PT, the uninfected untreated dogs (36.66 ± 3.12 µmol/L) had a significantly ($P < 0.01$) higher Cu^{2+} concentration, compared to the concentrations obtained in the infected untreated dogs (19.31 ± 0.77 µmol/L); and infected + ALA dogs (19.80 ± 0.73 µmol/L), respectively. There was no significant ($P > 0.05$) difference in Cu^{2+} concentrations at day 43 PT between the groups.

4.8.2 Effect of treatments on serum zinc concentration

Zinc (Zn^{+}) concentration (Figure 4.39) within the groups at base-line, days 11 PI, PT 8, 15 and 43, respectively did not differ. There was no significant ($P > 0.05$) difference in the Zn^{+} concentration at base-line, days 11 PI, 8 PT and 15 PT respectively. At day 43 PT, the Zn^{+} concentration in the infected + ALA dogs (87.68 ± 29.05 µmol/L) was significantly ($P < 0.001$) higher than the concentrations obtained in the uninfected untreated dogs (35.82 ± 6.69 µmol/L); infected untreated dogs (37.91 ± 4.51 µmol/L); infected + imidocarb dogs (23.82 ± 0.88 µmol/L); and infected + imidocarb + ALA dogs (38.92 ± 10.59 µmol/L), respectively.

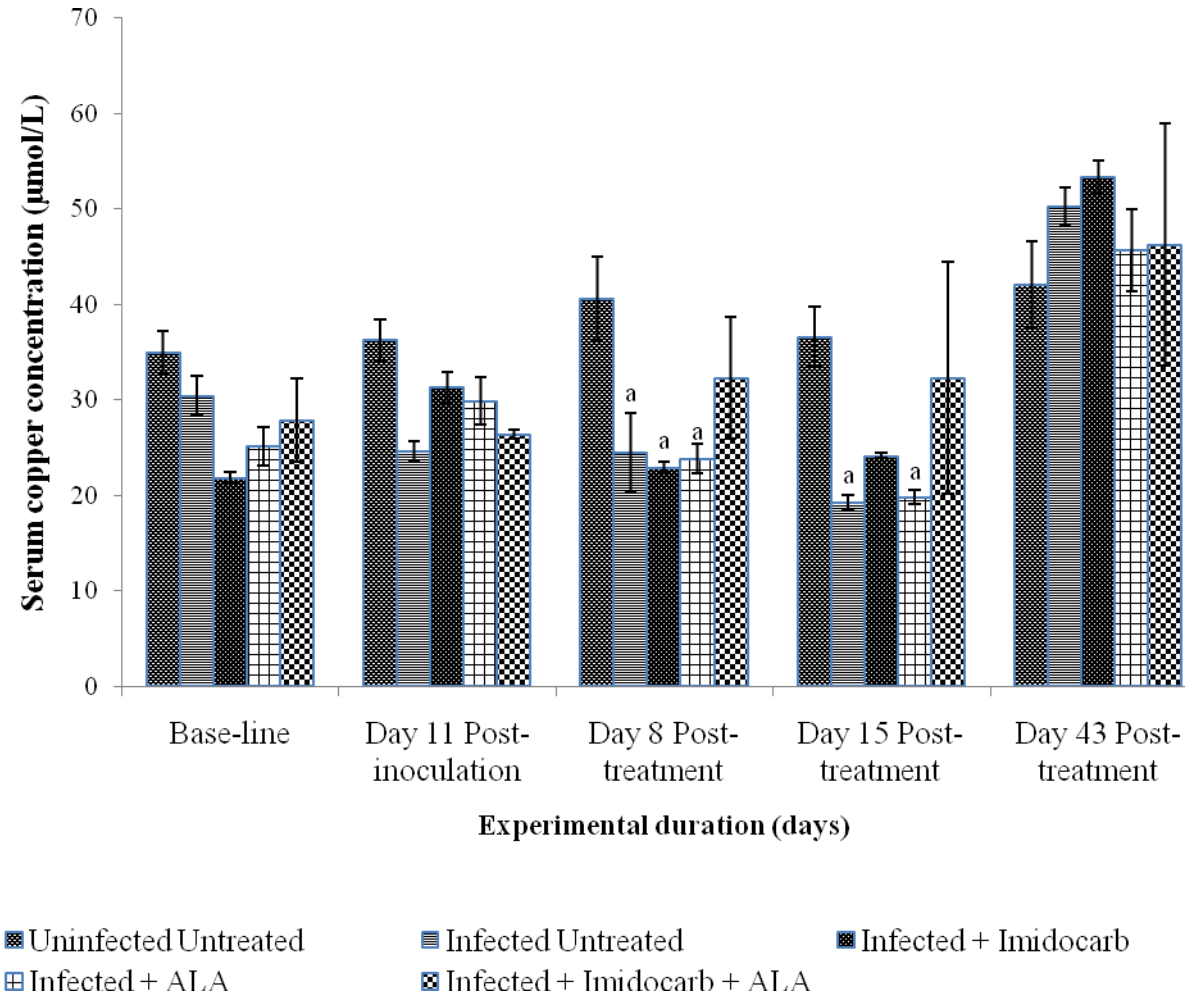


Figure 4.38: Effect of treatments on serum copper concentration in Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli*

[a]$P < 0.05$ versus uninfected untreated group

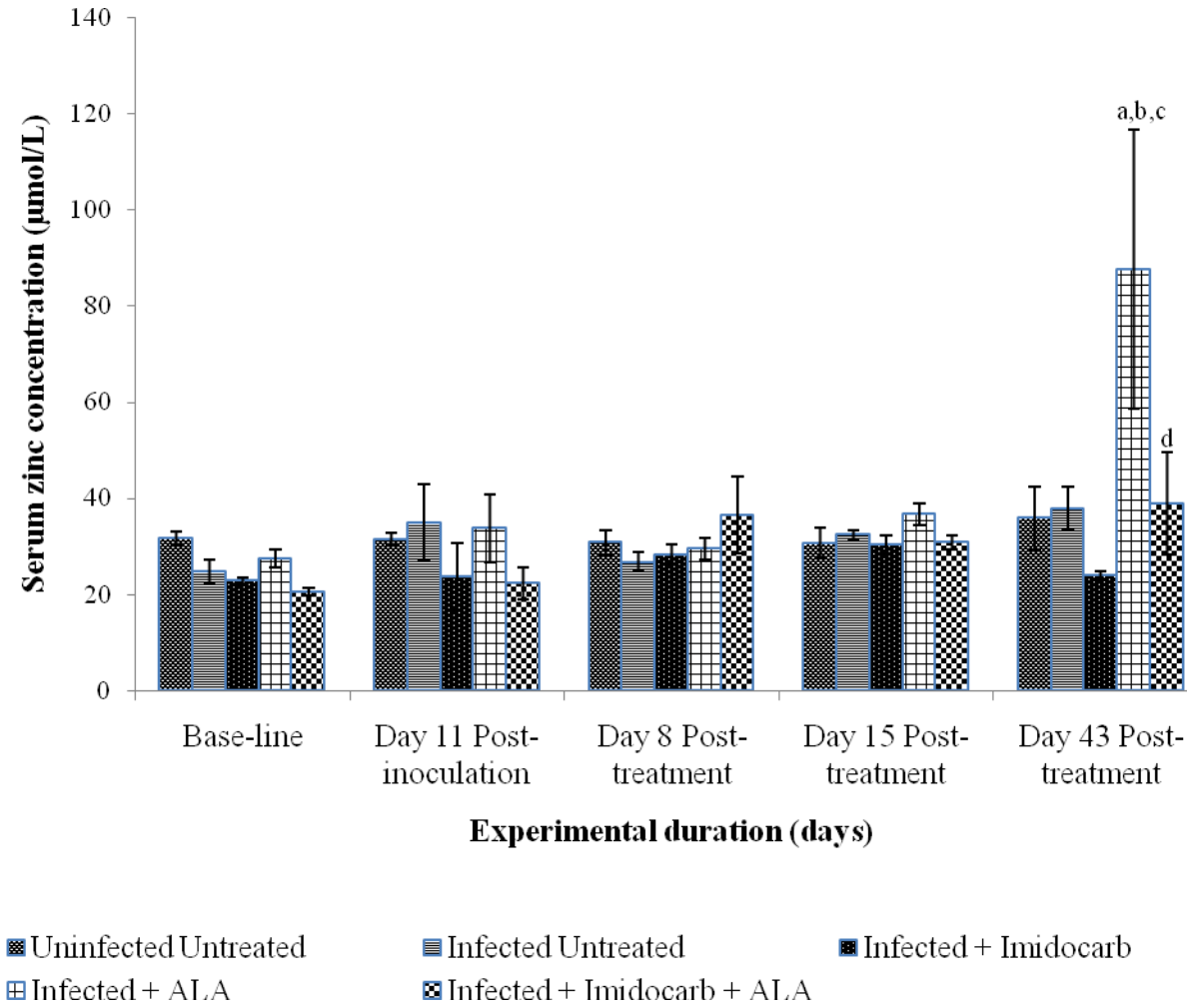


Figure 4.39: Effect of treatments on serum zinc concentration in Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli*

[a,b,c,d]P < 0.05 versus uninfected untreated, infected untreated, infected + imidocarb and infected + imidocarb + ALA groups, respectively

4.8.3 Effect of treatments on serum iron concentration

The iron (Fe^{2+}) concentrations (Figure 4.40) within the groups at base-line, day 11 PI, 8 PT, 15 PT and 43 PT, respectively were insignificant. At base-line, the uninfected untreated dogs (0.04 ± 0.00 mmol/L) had a lower ($P < 0.05$) iron concentration compared to the infected + ALA dogs (0.30 ± 0.04 mmol/L). At day 11 PI, the Fe^{2+} concentration in the uninfected untreated dogs (0.04 ± 0.00 mmol/L) was significantly ($P < 0.05$) lower than in the infected untreated dogs (0.20 ± 0.03 mmol/L); infected + imidocarb dogs (0.24 ± 0.05 mmol/L); infected + ALA dogs (0.20 ± 0.01 mmol/L); and infected + imidocarb + ALA dogs (0.21 ± 0.04 mmol/L), respectively. At day 8 PT, the Fe^{2+} concentration in the infected + imidocarb + ALA dogs (0.36 ± 0.05 mmol/L) was higher ($P < 0.001$) compared to the uninfected untreated dogs (0.04 ± 0.00 mmol/L); infected untreated dogs (0.12 ± 0.03 mmol/L); infected + imidocarb dogs (0.09 ± 0.04 mmol/L); infected + ALA dogs (0.18 ± 0.06 mmol/L;$P < 0.01$), respectively. At day 15 PT, the uninfected untreated dogs (0.04 ± 0.00 mmol/L) had a lower ($P < 0.01$) Fe^{2+} concentration compared to the infected untreated dogs (0.23 ± 0.04 mmol/L) and the infected + ALA dogs (0.22 ± 0.04 mmol/L), respectively. There was no significant ($P > 0.05$) difference in Fe^{2+} concentration between the groups at day 43 PT.

4.9 Effect of Treatments on Oxidative Stress Biomarkers

4.9.1 Effect of treatments on erythrocyte malondialdehyde concentration

Erythrocyte malondialdehyde (MDA) concentrations (Figure 4.41) within the uninfected untreated dogs were insignificant. In the infected untreated dogs, there was significant ($P < 0.01$) decrease MDA concentration at base-line (0.84 ± 0.29 µM), compared to day 11 PI (2.13 ± 0.23 µM). The MDA concentration at day 11 PI (2.13 ± 0.23 µM) was significantly ($P < 0.05$) lower than values recorded at PT days 8 (2.59 ± 0.14 µM); 15 (3.00 ± 0.42 µM;

P < 0.001); and 43 (6.68 ± 0.22 µM; P < 0.01), respectively. In the infected + imidocarb dogs, the MDA concentration was lower (P < 0.05) at day 11 PI (1.72 ± 0.21 µM) compared to day 15 PT (3.09 ± 0.89 µM).

In the infected + ALA dogs, a significant (P < 0.001) increase in MDA concentration was recorded at day 11 PI (2.52 ± 0.34 µM) compared to days 8 PT (2.11 ± 0.25 µM) and 15 PT (2.30 ± 0.30 µM; p < 0.01); but had a significant (P < 0.05) decrease compared to day 43 PT (8.61 ± 1.42 µM), respectively. In the infected + imidocarb + ALA dogs, a significant (P < 0.05) decrease in MDA concentration was recorded at day 11 PI (2.13 ± 0.24 µM) compared to values obtained at days 8 PT (2.53 ± 0.30 µM); 15 PT (2.14 ± 0.33 µM); and 43 PT (7.74 ± 1.54 µM), respectively. At day 11 PI, there was a significant (P < 0.05) decrease in MDA concentration in the uninfected untreated dogs (0.43 ± 0.04 µM), compared to the infected untreated (2.13 ± 0.23 µM), infected + imidocarb dogs (1.72 ± 0.21 µM); infected + ALA (2.52 ± 0.34 µM) and infected + imidocarb + ALA (2.13 ± 0.24 µM) dogs. At day 8 PT, there was a significant (P < 0.001) decrease MDA concentration in the uninfected untreated dogs (0.43 ± 0.12 µM) compared to the infected untreated dogs (2.59 ± 0.14 µM); infected + imidocarb dogs (2.43 ± 0.90 µM); infected + ALA dogs (2.11 ± 0.25 µM); and infected + imidocarb + ALA dogs (2.53 ± 0.30 µM), respectively. At day 15 PT, there was a lower (P < 0.001) MDA concentration in the uninfected untreated (0.45 ± 0.06 µM) compared to the values recorded in the infected untreated dogs (3.00 ± 0.42 µM); infected + imidocarb dogs (3.09 ± 0.89 µM); infected + ALA dogs (2.30 ± 0.30 µM), respectively. At day 43 PT, there was a significant (P < 0.001) decrease in MDA concentration in the uninfected untreated dogs (0.48 ± 0.06 µM), compared to dogs in the infected untreated (6.68 ± 0.22 µM); infected + imidocarb (6.55 ± 0.96 µM); infected + ALA (8.61 ± 1.42 µM); infected + imidocarb + ALA dogs (7.74 ± 1.54 µM), respectively.

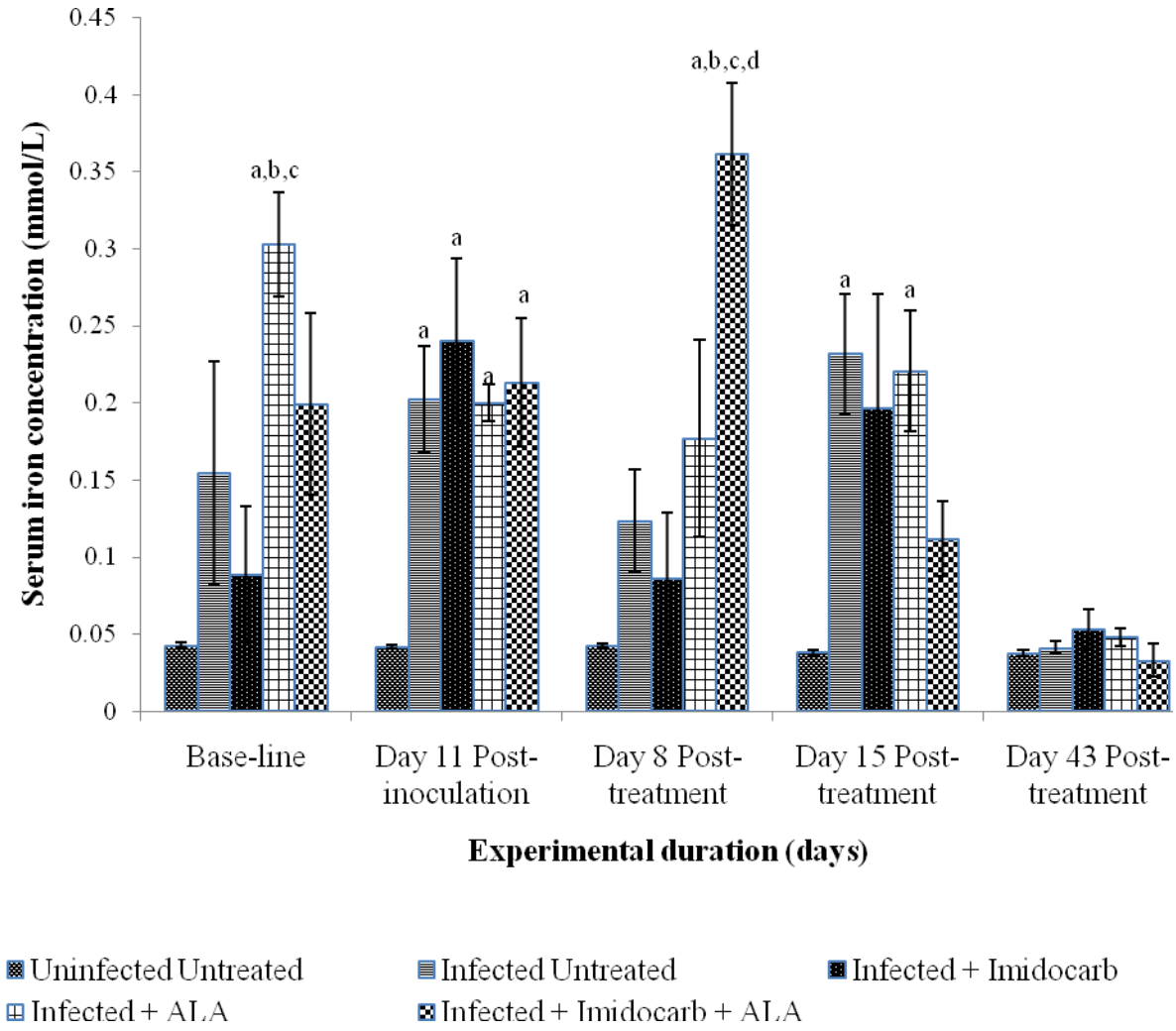


Figure 4.40: Effect of treatments on serum iron concentration in Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli*

$^{a,b,c,d}P < 0.05$ versus uninfected untreated, infected untreated, infected + imidocarb and infected + imidocarb + ALA groups, respectively

Normal range: 10 - 30 mmol/L

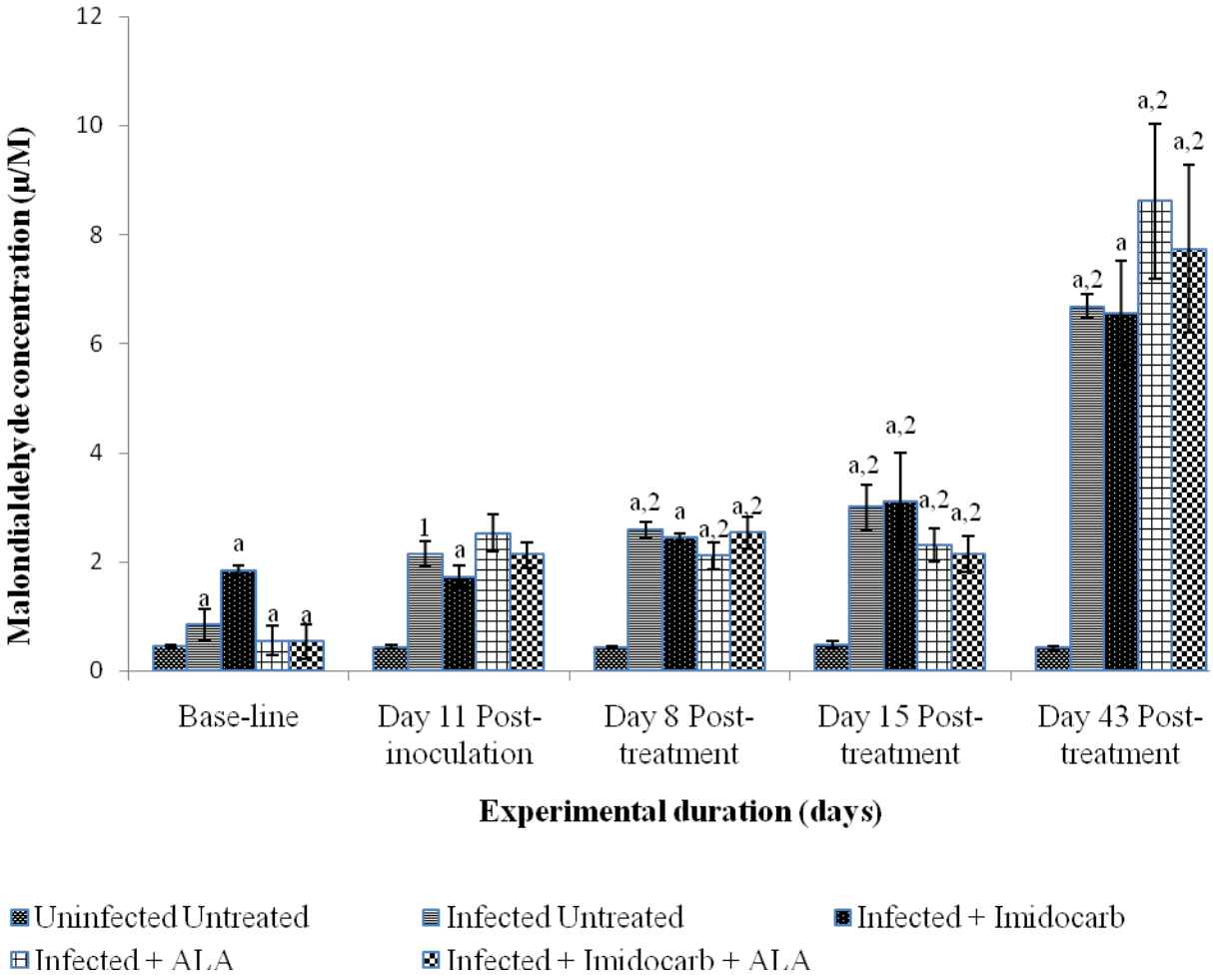


Figure 4.41: Effect of treatments on erythrocyte malondialdehyde concentration in Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli*

[1,2]P < 0.05 versus base-line and day 11 PI
[a]P < 0.05 versus uninfected untreated group

4.9.2 Effect of treatments on platelet glutathione peroxidase

The infected untreated dogs had a higher ($P < 0.001$) platelet glutathione peroxidase (GPx) activity (Figure 4.42) at base-line (117.94 ± 8.17 U/L), compared to days 11 PI (6.73 ± 1.31 U/L); 8 PT (63.96 ± 0.53 U/L); 15 PT (22.50 ± 3.44 U/L); and 43 PT (10.18 ± 2.25 U/L), respectively. There was a significant ($P < 0.001$) increase in GPx activity at day 8 PT (63.96 ± 0.53 U/L) compared to activities recorded at days 11 PI (6.73 ± 1.31 U/L); 15 PT (22.50 ± 3.44 U/L); and 43 PT (10.18 ± 2.25 U/L), respectively. In the infected + imidocarb dogs, the GPx activity at base-line (104.73 ± 23.27 U/L) was higher ($P < 0.05$) than at days 11 PI (9.46 ± 2.73 U/L); 15 PT (9.67 ± 1.68 U/L); and 43 PT (24.60 ± 4.42 U/L), respectively. In the infected + ALA dogs, the base-line (67.58 ± 6.18 U/L) GPx activity was significantly ($P < 0.001$) higher than those recorded at days 11 PI (5.61 ± 0.78 U/L); 15 PT (21.17 ± 2.04 U/L); and 43 PT (10.52 ± 1.11 U/L), respectively. There was a significant ($P < 0.001$) increase in GPx activity at day 8 PT (51.94 ± 6.24 U/L) compared to days 11 PI (11.16 ± 2.86 U/L); 15 PT (21.17 ± 2.04 U/L); and 43 PT (10.52 ± 1.11 U/L), respectively.

In the infected + imidocarb + ALA dogs, the base-line (121.55 ± 0.84 U/L) GPx activity was significantly ($P < 0.001$) increased compared to activites obtained at days 11 PI (5.61 ± 0.78 U/L); 8 PT (60.99 ± 3.63 U/L); 15 PT (13.46 ± 2.11 U/L); and 43 PT (13.25 ± 1.47 U/L), respectively. Higher ($P < 0.001$) GPx activity was recorded at day 8 PT (60.99 ± 3.63 U/L) compared to days 11 PI (5.61 ± 0.78 U/L); 15 PT (13.46 ± 2.11 U/L); and 43 PT (13.25 ± 1.47 U/L), respectively. Higher ($P < 0.01$) GPx activity was recorded at base-line in the infected untreated dogs (117.94 ± 8.17 U/L), compared to the activites recorded in the uninfected untreated dogs (91.12 ± 7.24 u/L); infected + ALA dogs (67.58 ± 6.18 U/L; $P < 0.001$). There was a significantly ($P < 0.01$) higher GPx activity in the infected + imidocarb dogs (104.73 ± 23.27 U/L), compared to the activity in the infected + ALA dogs

(67.58 ± 6.18 U/L). The GPx activity was lower ($P < 0.01$) in the infected + ALA dogs (67.58 ± 6.18 U/L), compared to those recorded in the infected + imidocarb dogs (104.73 ± 23.27 U/L) and infected + imidocarb + ALA dogs (121.55 ± 0.84 U/L), respectively. At day 11 PI, the GPx activity was higher ($P < 0.001$) in the uninfected untreated dogs (91.12 ± 7.24 U/L), compared to the infected untreated dogs (6.73 ± 1.31 U/L); infected + imidocarb dogs (9.46 ± 2.73 U/L); infected + ALA dogs (11.16 ± 2.86 U/L); and infected + imidocarb + ALA dogs (5.61 ± 0.78 U/L), respectively. At day 8 PT, there was a significant ($P < 0.05$) increase GP_X activity in the uninfected untreated dogs (88.65 ± 7.65 U/L), compared to the infected untreated dogs (63.96 ± 0.53 U/L); infected + ALA dogs (51.94 ± 6.24; $P < 0.001$); and infected + imidocarb + ALA dogs (60.99 ± 3.63 U/L; $P < 0.05$), respectively. At day 15 PT, higher ($P < 0.001$) GPx activity was recorded in the uninfected untreated dogs (88.31 ± 7.75 U/L), compared to the infected untreated dogs (22.50 ± 3.44 U/L); infected + imidocarb dogs (9.67 ± 1.68 U/L); infected + ALA dogs (21.17 ± 2.04 U/L); and infected + imidocarb + ALA dogs (13.46 ± 2.11 U/L). At day 43 PT, there was a higher ($P < 0.001$) GPx activity recorded in the infected untreated dogs (22.50 ± 3.44 U/L) was higher than that obtained in the infected + imidocarb dogs (9.67 ± 1.68 U/L); infected + ALA dogs (10.52 ± 1.11 U/L); and infected + imidocarb + ALA dogs (13.25 ± 1.47 U/L), respectively.

4.9.3 Effect of treatments on serum superoxide dismutase

In the infected untreated dogs, base-line (1.97 ± 0.18 U/L) superoxide dismutase (SOD) activity (Figure 4.43) was lower ($P < 0.01$) compared to day 11 PI (2.90 ± 0.22 U/L); but, subsequently, the activity was higher ($P < 0.01$) than at PT days 8 (1.08 ± 0.10 U/L); and 15 (0.33 ± 0.05 U/L). The SOD activity recorded at day 11 PI (2.90 ± 0.22 U/L) increased ($P < 0.001$) compared to activities obtained at PT days 8 (1.08 ± 0.10 U/L); 15 (0.33 ± 0.05 U/L); and 43 PT (1.83 ± 0.11 U/L), respectively. There was significantly ($P < 0.01$) lower

SOD activity at day 15 PT (0.33 ± 0.05 U/L) compared to PT days 8 (1.08 ± 0.10 U/L); and 43 (1.83 ± 0.11 U/L), respectively.

In the infected + imidocarb dogs, lower ($P < 0.05$) base-line (2.12 ± 0.29 U/L) GPx activity was recorded compared to days 11 PI (3.01 ± 0.05 U/L), but there was lower ($P < 0.01$) SOD activity at day 8 PT (1.10 ± 0.15 U/L); and 15 PT (0.70 ± 0.06 U/L; $P < 0.001$). The SOD activity at day 43 PT (2.09 ± 0.11U/L) was significantly ($P < 0.01$) higher than days 8 PT (1.10 ± 0.15 U/L) and 15 PT (0.70 ± 0.06 U/L; $P < 0.001$), respectively. The infected + ALA dogs had a lower ($P < 0.05$) SOD activity at base-line (2.14 ± 0.20 U/L) compared to day 11 PI (2.93 ± 0.32 U/L), but decreased ($P < 0.05$) SOD activity was recorded compared to days 8 PT (1.19 ± 0.06 U/L) and 15 PT (0.59 ± 0.04 U/L; $P < 0.001$). There was a significant ($P < 0.001$) increase in SOD activity at days 11 PI (2.93 ± 0.32 U/L) and 43 PT (2.28 ± 0.11 U/L), compared to activities recorded at PT days 8 (1.19 ± 0.06 U/L), and 15 (0.59 ± 0.04 U/L). The infected + imidocarb + ALA dogs had a higher ($P < 0.05$) SOD activity at base-line (2.14 ± 0.39 U/L), days 11 PI (2.71 ± 0.31 U/L) and 43 PT (2.17 ± 0.23 U/L) compared to PT days 8 (0.95 ± 0.95 U/L; $P < 0.01$) and 15 (0.36 ± 0.06 U/L; $P < 0.001$), respectively. At day 8 PT, there was a significant ($P < 0.001$) increase in the SOD activity in the uninfected untreated dogs (2.38 ± 0.14 U/L) compared to the infected untreated dogs (1.08 ± 0.10 U/L); infected + imidocarb dogs (1.10 ± 0.15 U/L; $P < 0.01$); infected + ALA dogs (1.19 ± 0.06 U/L); and infected + imidocarb + ALA dogs (0.95 ± 0.95 U/L), respectively. At day 15 PT, the uninfected untreated dogs (1.28 ± 0.47 U/L) had a higher ($P < 0.01$) SOD activity compared to the infected untreated dogs (0.33 ± 0.05 U/L) and infected + imidocarb + ALA dogs (0.36 ± 0.06 U/L; $P < 0.05$). At day 43 PT, there was a significantly ($P < 0.05$) lower SOD activity in the uninfected untreated dogs (1.17 ± 0.53 U/L) compared to the infected + imidocarb dogs (2.09 ± 0.11 U/L); infected + ALA dogs

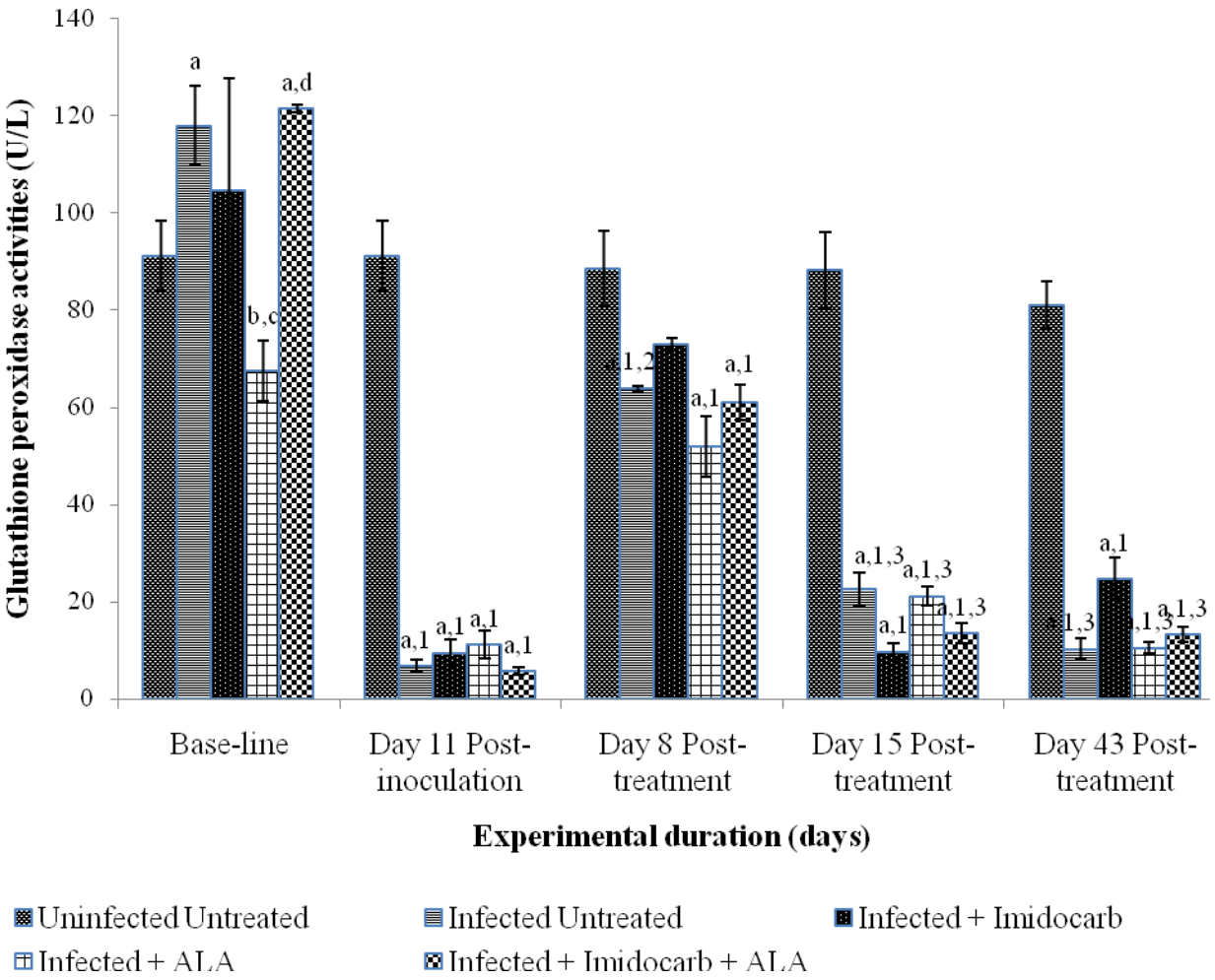


Figure 4.42: Effect of treatments on platelets glutathione peroxidase in Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli*

[1,2,3]P < 0.05 versus base-line, days 11 PI and 8 PT, respectively
[a,b,c,d]P < 0.05 versus uninfected untreated, infected untreated infected + imidocarb and infected + imidocarb + ALA groups, respectively

(2.28 ± 0.11 U/L; P < 0.01); and infected + imidocarb + ALA dogs (2.17 ± 0.23 U/L), respectively.

4.10 Effect of Treatments on Tumour Necrosis Factor-Alpha

In the uninfected untreated group, there was significantly (P < 0.05) lower tumour necrosis factor-α (TNF-α) (Figure 4.44) at day 11 PI (35.77 ± 4.20 pg/mL) compared to day 15 PT (40.92 ± 5.28 pg/mL). There was no significant (P > 0.05) difference within the infected untreated, infected + imidocarb and infected + imidocarb + ALA dogs. Lower (P < 0.05) TNF-α was recorded between the uninfected untreated dogs (35.77 ± 4.20 pg/mL; 40.11 ± 5.28 pg/mL; 40.92 ± 5.28 pg/mL) and infected + imidocarb dogs (230.31 ± 21.61 pg/mL; 230.19 ± 70.56 pg/mL; 272.74 ± 73.48 pg/mL) at days 11 PI, 8 PT and 15 PT, respectively.

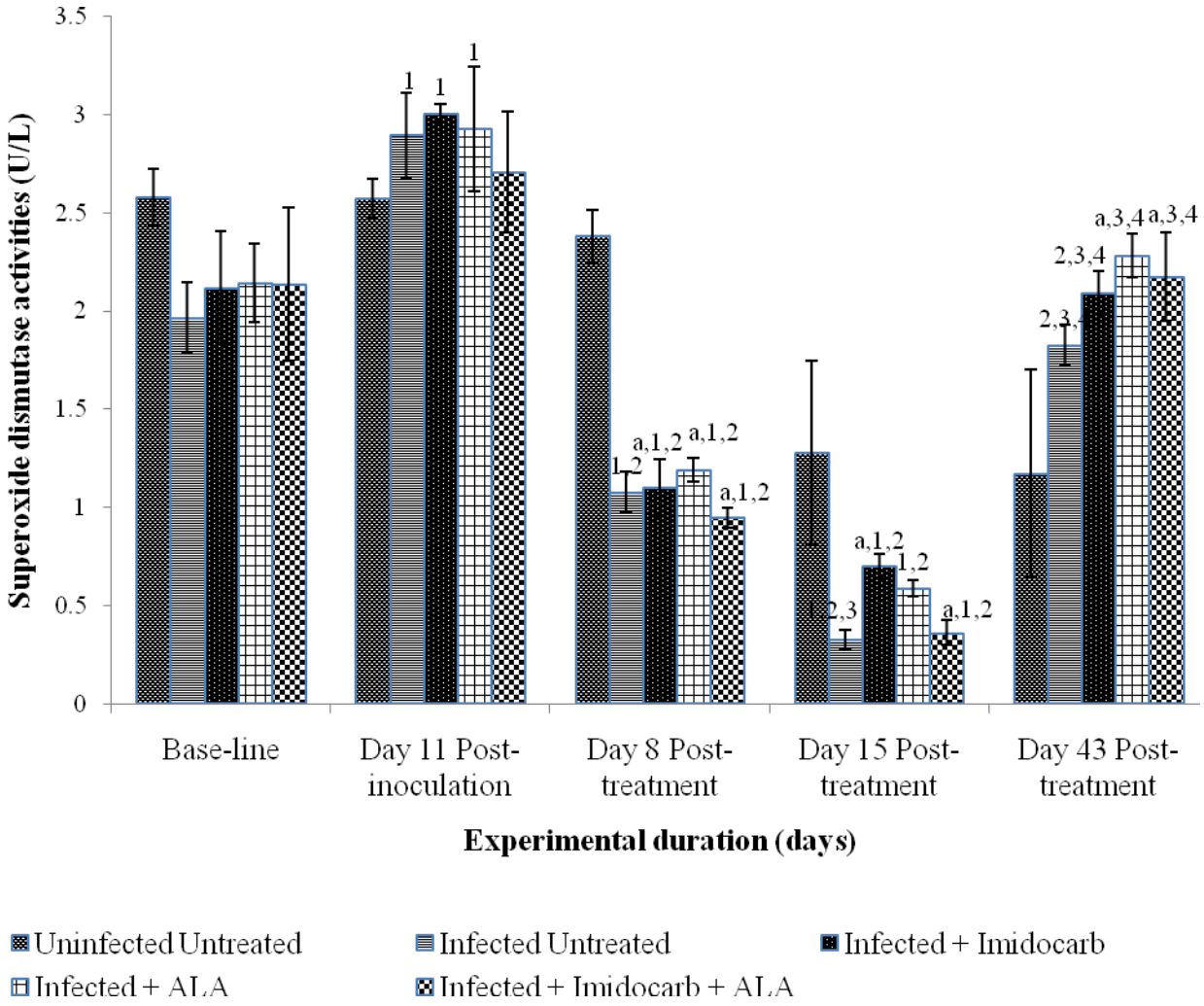


Figure 4.43: Effect of treatments on serum superoxide dismutase activity in Nigerian indigenous dogs experimentally infected with Babesia canis vogeli

[1,2,3,4]P < 0.05 versus base-line, days 11 PI 8 PT and 15 PT respectively
[a]P < 0.05 versus uninfected untreated group

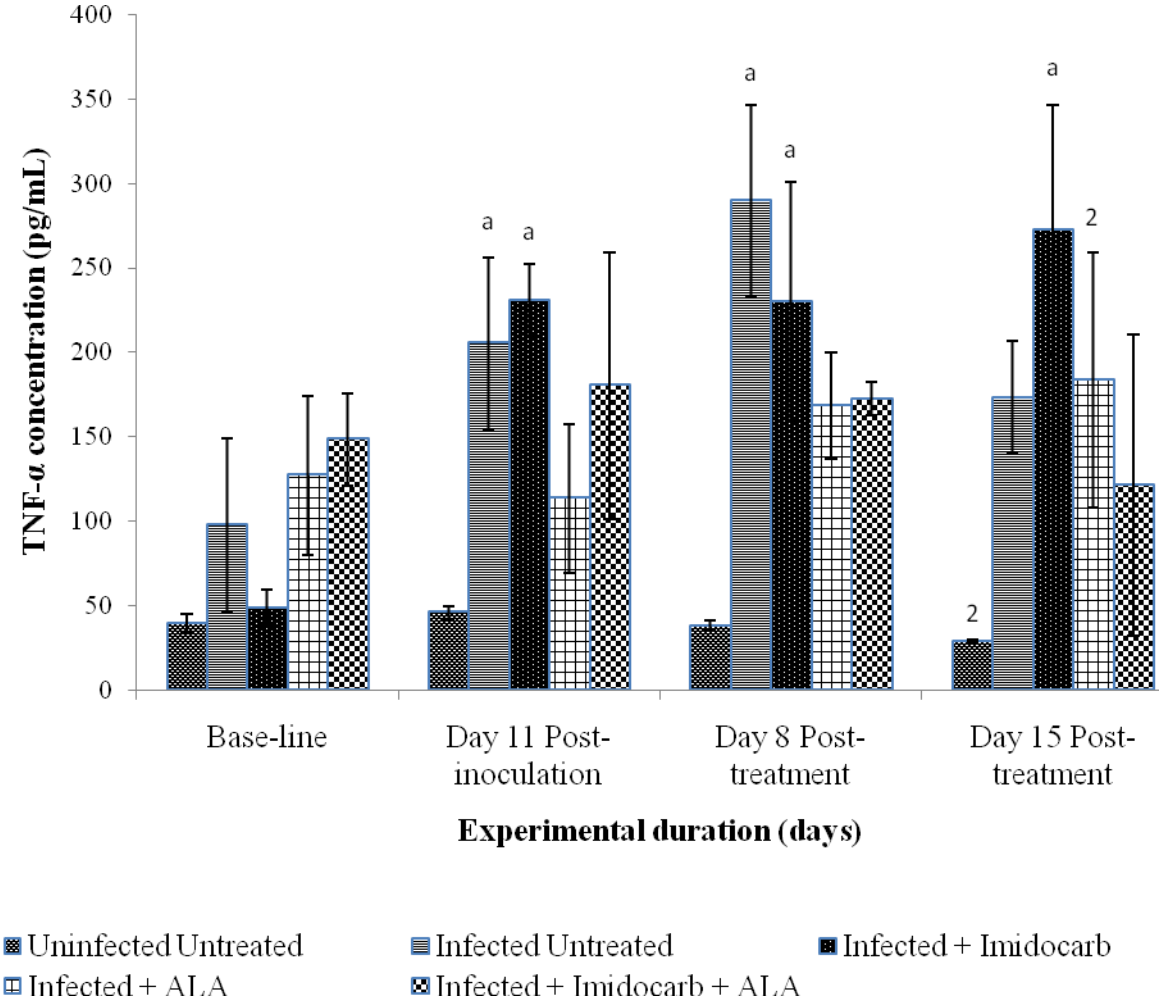


Figure 4.44: Effect of treatments on serum tumour necrosis factor-α in Nigerian indigenous dogs experimentally infected with *Babesia canis vogeli*

[2]P < 0.05 versus day 11
[a]P < 0.05 versus uninfected untreated group

CHAPTER FIVE

5.0 DISCUSSION

Molecular identification and characterisation of the inoculated *Babesia* spp. indicated *Babesia canis vogeli*. This may be attributed to the presence of *Rhipicephalus sanguineous* in Nigeria, which is the vector that transmits *B. c. vogeli* (Halos *et al.,* 2013; Penzhorn, 2016). *Babesia c. vogeli* has been reported by previous authors (Sasaki *et al.,* 2007; Ogo *et al.,* 2011; Shima *et al.,* 2015; Cardoso *et al.,* 2016) to be endemic in Nigeria. The clinical manifestations of canine babesiosis depend on the virulence of the *Babesia* spp., host's factors, including age, and immune status (Andoni *et al.,* 2012). The mild clinical manifestations observed in the present study further confirmed the presence of *B. c. vogeli* and agrees with the findings of previous authors (Halos *et al.,* 2013; Solano-Gallego *et al.,* 2016), who reported that *Babesia canis vogeli* is the least pathogenic among the three strains of *Babesia canis* sub spp and causes sub-clinical, mild or moderate clinical manifestations in adult dogs. The generation of macrophage-produced mediators may explain the clinical manifestations in babesiosis, which include fever, anorexia, myalgia, reported in humans (Vannier *et al.,* 2008), and animals (Solano-Gallego *et al.,* 2016). In the present study, the clinical manifestations observed included fever, which was observed at different days during the course of the infection, as early as day 2 PI to day 3 PT. This finding may be attributed to the release of endogeneous pyrogens from lysed RBC, parasitic destruction, activation of inflammatory mediators and generation of ROS often associated with canine babesiosis. The findings have been reported by Salem and Farag (2014) and Bharath *et al.* (2016). Loss of appetite was observed in all the groups at day 0 PT, which may be due to the increased generation of TNF-α and IL-1 that usually occur during babesiosis (Yan *et al.,* 2014). The pale mucous membranes observed in this study may be due to haemolysis that occurs in babesiosis as a result of tissue hypoxia. The hypoxia,

apparently, triggers tissue injury due to the release of immunological factors (cytokines, free radicals, nitric oxide and other inflammatory mediators), causing systemic inflammatory response syndrome (Aleri *et al.*, 2011). The absence of dry muzzle, ocular discharge and myalgia in the infected + imidocarb + ALA dogs may be attributed to the antiprotozoal, anti-inflammatory and antioxidant properties of combination imidocarb and ALA.

Dullness and dry muzzle recorded at day 0 PT in all the groups fell within the incubation period of canine babesiosis of 10 - 21 days. This may be due to hypoxia that occurred as a result of anaemia, associated with the release of cytokines in systemic inflammatory response syndrome (SIRS) in canine babesisois (Bharath *et al.*, 2016). The clinical manifestations observed in this study are consistent with the findings of several authors (Adaszek *et al.*, 2011; Rene-Martellet *et al.*, 2013; Sivajothi *et al.*, 2014; Yao *et al.*, 2014; Davitkov *et al.*, 2015; Nalubamba *et al.*, 2015), who reported anorexia, fever, pale mucous membrane and splenomegaly in canine babesiosis. This is contrary to the findings of Bharath et al. (2016) who reported congested mucous membrane in dogs with babesiosis. The splenomegaly recorded was as a result of sequestration of *Babesia* parasites in the spleen (Rene-Martellet *et al.*, 2013). The splenomegaly (100%) observed in the infected untreated dogs 2 weeks post-treatment indicated the occurrence of sequestration of *B. c. vogeli* in the spleen. This indicates an on-going erythrophagocytosis of the parasitised RBCs by macrophages in the spleen. The result agrees with the findings of previous authors (Aysul *et al.*, 2013; Salem and Farag, 2014; Koster *et al.*, 2015), who reported that splenomegaly occurs in canine babesiosis. The asplenomegaly observed in the infected + imidocarb + ALA group post-treament may be attributed to the role of ALA as a cytoprotectant antioxidant (Kim *et al.*, 2013).

An increased RT of dogs in the infected untreated dogs though within the normal range (37.6°C - 39.5°C) at days 11 PI and 15 PT compared to day 43 PT may be due to the parasitaemia and lysis of the RBC by *B. c. vogeli*, which releases pyrogens that eventually induced fever. The rise in temperature might also be an attempt by the dogs to overcome or eradicate the parasites. This result agrees with the findings of Nalumbamba *et al.* (2015) who reported a rise in RT in *Babesia*-infected dogs in Zambia. Fever (33.3%) was recorded at day 6 PI in the infected + imidocarb dogs, which ceased after treatment. The rise in RT in the infected + imidocarb dogs at base-line and day 11 PI compared to PT days 15 and 43 PT improved considerably after treatment with imidocarb dipropionate. This finding suggests that imidocarb dipropionate may eradicate the *Babesia canis vogeli* from circulation. The result agrees with the findings of Salem and Farag (2014), who reported that the administration of two doses of imidocarb dipropionate (2 weeks apart) effectively cleared *B. canis* spp. from circulation. In the infected + ALA dogs, the onset of fever (60%) was at day 3 PI, with an increased RT in the infected + ALA dogs at PT days 8 and 15 compared to day 43 PT. This indicates that ALA administered singly did not eradicate *Babesia canis vogeli* from circulation. Dogs in the infected + imidocarb + ALA had 33.3% fever, which was observed at day 3 PI. The elevated RT in the infected + imidocarb + ALA dogs at base-line compared to day 43 PT indicated that co-administration of imidocarb and ALA was able to eradicate the parasite, and also ameliorated the effect of oxidative stress that accompanies canine babesiosis. The result agrees with the findings that the clinical manifestations observed in canine babesiosis are induced as a result of oxidative stress (Zhang *et al.*, 2011), ameliorated by ALA administration. The insignificant ($P > 0.05$) changes at day 11 PI between the infected groups and the uninfected untreated group indicated that normal RT values were recorded in the infected groups at day 11 PI. This finding may be attributed to the transient parasitaemia that occurs in canine babesiosis, thus

which agrees with the findings of Nalumbamba *et al.* (2015), who reported transient parasitaemia in dogs with babesiosis in Zambia. The RT values obtained in the present study were all within the normal range; although fever was recorded in individual dogs, in different groups, at different times, during the course of the infection. This variation may be due to the infectivity of the parasite, time of blood collection, stage of the parasitaemia and the ambient temperature. Although at day 8 PT, the RT increased in the infected + ALA and infected + imidocarb + ALA groups, compared to the uninfected untreated group, the increase may be due to the inability of ALA alone to eradicate the *Babesia* spp. from circulation, and the oxidative stress that occurs in canine babesiosis. The decrease in RT of dogs in the infected untreated and infected + imidocarb compared to the infected + ALA dogs, was apparently due to the low infectivity *of Babesia canis vogeli* in the infected untreated dogs and the ability of imidocarb dipropionate to eradicate parasitaemia in large *Babesia* infection (Salem and Farag, 2014). The result agrees with the findings of Torbica *et al.* (2013), who reported a decrease in RT a day after antibabesial treatment. The high RT recorded in the infected untreated and infected + ALA dogs compared to the uninfected untreated dogs at day 15 PT, may be due to haemolysis by *Babesia canis vogeli* which released pyrogens inducing fever. The result agrees with the findings of Nalumbamba *et al.* (2015) who reported an increase in RT in Zambian dogs infected with babesiosis. The lower RT recorded in the infected + imidocarb and infected + imidocarb + ALA dogs compared to the infected untreated and infected + ALA dogs, indicated that two doses of imidocarb administered two weeks apart were able to eradicate the clinical manifestations associated with canine babesiosis (Salem and Farag, 2014). It may also be due to the anti-inflammatory and antioxidant (Zhang *et al.*, 2011) properties of a combination of imidocarb and ALA in the treatment of babesiosis in dogs. The result agrees with the findings of Gomes and Negrato (2014), who reported the anti-inflammatory and antioxidant properties

of ALA as a therapeutic agent in diabetes and other chronic diseases. The increased RT in the infected + ALA dogs compared to the infected + imidocarb dogs at day 43 PT may be due to the presence of *Babesia canis vogeli*. The slight decrease in RT of dogs treated with imidocarb and ALA, compared to the uninfected untreated dogs, may be attributed to the synergistic effect of imidocarb and ALA in the treatment of canine babesiosis. This result is similar with the findings of Gonzalez-Perez *et al.* (2008) and Tesfaye *et al.* (2011), who reported the synergistic effect of ALA and vitamin E in the amelioration of symptoms of diabetic neuropathy.

The insignificant ($P > 0.05$) difference in the PR at day 11 PI indicated that infection with *B. c. vogeli* do not affect pulse rate.The elevated PR above normal in the infected + ALA group compared to uninfected untreated, infected + imidocarb and infected + imidocarb + ALA groups at day 8 PT may be due to oxidative stress, associated with canine babesiosis. Hypoxic anaemia in babesiosis results in elevated PR and RR, a compensatory mechanism by the host's body to oxygenate the tissues. The result agrees with the findings of previous authors (Gonde *et al.,* 2014; Konto *et al.,* 2014) that elevated PR occurs in dogs with babesiosis. The result also confirms the finding of Adebayo *et al.* (2016), who reported a significant increase in PR and heart rate that may be common findings in uncomplicated canine babesiosis (Torbica *et al.*,2013), and sign of cardiac pathology. Similarly, the finding might also be the reason for the increase in PR in the infected untreated dogs compared to the infected + imidocarb + ALA dogs. It may also be as a result of the ameliorative roles of imidocarb and ALA in the treatment of dogs with babesiosis. The elevated PR recorded in the infected untreated and the infected + ALA dogs at day 15 PT, compared to the uninfected untreated dogs, may be associated with the compensatory response of the dogs to anaemic hypoxia, caused by haemolysis of the RBCs during

Babesia canis vogeli infection. Similarly, the rise in the PR in the infected + ALA group compared to the infected + imidocarb, and infected + imidocarb + ALA groups, may be due to the ability of imidocarb administered singly or its co-administration with ALA in the amelioration of the oxidative stress, associated with canine babesiosis. This finding may be explained by the decrease obtained in PR in the infected + imidocarb + ALA dogs, compared to the infected untreated dogs.

In the present study, the decrease in RR at PT days 8 and 43 compared to the base-line RR in the infected untreated group; decreased RR at day 43 PT compared to the base-line in the infected + ALA group; and the increase in RR at days 11 PI and 43 PT compared to the base-line indicated that respiratory impairment was not involved in *B. c. vogeli* infection. This observation was further supported by the insignificant ($P > 0.05$) difference recorded between the groups at base-line, days 11 PI and PT days 8, 15, 43, respectively. The result agrees with the findings of Adebayo *et al.* (2016), who reported insignificant ($P > 0.05$) difference in the respiratory rates between *Babesia*-positive and -negative dogs. The respiratory rates obtained in this study were all within the normal respiratory range of 18 - 34 cycles/min (Adebayo *et al.*, 2016) during the course of the infection.

The weight gain at day 11 PI, compared to the base-line, and weight loss at post-treatment in the infected untreated dogs may be attributed to the weakness, lethargy, loss of appetite and drop in weight that are often reported in canine babesiosis (Spolidorio *et al.*, 2011; Kumar *et al.*, 2015; Nalubamba *et al.*, 2015; Solano-Gallego *et al.*, 2016). The slight weight gain at day 11 PI recorded in the infected + ALA dogs compared to base-line, and the marked weight loss at post-treatment may be attributed to babesiosis and the role of ALA in weight loss (Gomes and Negrato, 2014). Similarly, the weight gain at day 11 PI compared

to the base-line and weight loss at post-treatment may be due to the role of ALA in weight loss. This finding may be attributed to some actions of ALA, including activation of 5′ adenosine monophosphate-activated protein kinase (AMPK) in peripheral and brain tissue (Ramamurthy and Ronnet, 2012), inhibition of nuclear factor -kappa β (Nf-Kβ) and adipocyte differentiation (Gomes and Negrato, 2014). The weight loss recorded in the infected untreated, infected + imidocarb, infected + ALA and infected + imidocarb + ALA groups at post-treatment compared to the uninfected untreated group, indicates that weight loss caused by *Babesia canis vogeli* may be evident, despite therapeutic interventions.

The decrease in PCV below the normal range of 37% - 55% (Nalubamba *et al.,* 2015) recorded in the present study at day 11 PI in the infected untreated, infected + imidocarb, infected + ALA and infected + imidocarb + ALA groups compared to the uninfected untreated group, indicated mild anaemia (30 - 37%) due to haemolysis (Nalubamba *et al.,* 2015; Piratae *et al.,* 2017). The haemolysis may be either intravascular or extravascular. This is in agreement with the findings of Nalubamba et al. (2015), who reported a decrease in PCV in dogs naturally infected with *B. rossi* in Zambia. This finding was confirmed by the pale ocular mucous membrane, observed in all the infected groups as early as day 6 PI. The normal PCV in the infected untreated compared to the uninfected untreated group at day 8 PT might be due to the movement of plasma from the vasculature to the extravascular as a result of dehydration. The dehydration was apparently caused by haemoconcentration, which is a complication of canine babesiosis (Mohr *et al.,* 2000; Schoeman, 2009; Kules *et al.,* 2014) in the infected untreated group. It may also be observed in hypoxic condition which can be physiological or pathological and a clinical manifestation observed in non-survivor dogs, infected with *B. c. rossi* (Goddard *et al.,* 2016). Similarly, dogs in the infected + imidocarb and infected + imidocarb + ALA were non-anaemic, but slight

anaemia was recorded in the infected + ALA dogs. The result demonstrated that single administration of imidocarb and its combination with ALA ameliorated the haemolysis in canine babesiosis. This is the first documentation of the use of ALA singly and in combination with imidocarb dipropionate in the treatment of canine babesiosis to the best of our knowledge.

The decrease in PCV recorded in the infected untreated, infected + imidocarb and infected + ALA groups at day 15 PT, compared to the uninfected untreated group, showed mild anaemia, in all the infected groups (Piratae *et al.,* 2017). The normal PCV obtained in the infected + imidocarb + ALA dogs indicates the synergistic effect of imidocarb and ALA in the treatment of *B. canis vogeli*. ALA regenerates vitamin E, which protects against DNA oxidative damage and lipid peroxidation of polyunsaturated fatty acids in the cell membrane, including that of the erythrocytes. Therefore ALA ameliorated (Gomes and Negrato, 2014) the effect of haemolysis that usually occurs in canine babesiosis. The normal PCV in all the infected dogs at day 43 PT, compared to the uninfected untreated indicated an effective therapy, with no relapse. It also indicated a regenerative anaemia which occurs in canine babesiosis, reported by previous authors (Zvorc *et al.,* 2010; Shah *et al.,* 2011; Gonde *et al.,* 2014; Koster, 2015; Solano-Gallego *et al.,* 2016). The result also agrees with the finding of Scheepers *et al.* (2011), who reported regenerative anaemia in transfused *B. rossi*-infected dogs within 2 - 3 weeks.

The decrease in RBC count in the infected untreated, infected + imidocarb, infected + ALA and infected + imidocarb + ALA dogs at day 11 PI, compared to the uninfected untreated dogs may be due to *B. c. vogeli*-induced haemolysis, which may either result into intravascular and/or extravascular haemolysis. Haemolysis in *B. c. vogeli*-infected dogs has

been reported by several authors (Fabisiak *et al.*, 2010; Salem and Farag, 2014; Solango-Gallego *et al.*, 2016). The anaemia observed in the infected untreated dogs at day 8 PT agrees with the finding of Salem and Farag (2014) who reported normal RBC count in 38.5% of *B. canis vogeli*-infected Egyptian dogs. The normal RBC count of dogs in the infected + imidocarb and infected + imidocarb + ALA dogs compared to the uninfected untreated dogs indicated an improvement after treatment; especially in the infected + imidocarb dogs, where the highest RBC count was recorded among the infected dogs. The result agrees with the findings Sikorski *et al.* (2010) and Vichova *et al.* (2016), who reported increased RBC count as evidence of clinical improvement after therapy in acute babesiosis. This finding also confirms the result obtained by Torbica *et al.* (2013) who reported elimination of parasitised RBCs 2 - 3 days after imidocarb administration. The result is also consistent with the findings of Irwin (2009) who reported protection of dogs from *Babesia canis* infection after administration of a single dose of imidocarb dipropionate (6 mg/kg). The normal RBC count in the infected + imidocarb and infected + imidocarb + ALA dogs may be due to the ameliorative, anti-inflammatory and antioxidant roles of imidocarb dipropionate and ALA administered either singly or in combination in the regeneration of RBC. The reduction in the RBC count in the infected + ALA dogs suggests anaemia, indicative that ALA alone was not able to prevent *Babesia canis vogeli*-induced RBC destruction. The decreased RBC count recorded at day 15 PT in the infected untreated, infected + imidocarb and infected + ALA dogs, compared to the uninfected untreated dogs indicated mild anaemia due to an on-going haemolysis. It may also be due to slow resorption rate of imidocarb dipropionate thus causing delayed recovery in the infected + imidocarb group (Torbica *et al.*, 2013). However, the infected + imidocarb + ALA dogs had normal RBC count, which showed there was neither anaemia nor relapse. This finding demonstrated the ameliorative role of administration of a combination of

imidocarb and ALA in the regeneration of RBC; consistent with the finding of El-Nakib *et al.* (2013) who reported the beneficial role of ALA as an adjunct to erythropoietin in the management of patients undergoing haemodialysis. The normal RBC counts in all the infected dogs (infected untreated, infected + imidocarb, infected + ALA and infected + imidocarb + ALA) at day 43 PT compared to the uninfected untreated dogs may suggest the sub-clinical/chronic form (Harvey *et al.,* 2017) of babesiosis or dehydration (Carli *et al.,* 2009), which is usually observed in *B. c. vogeli* infection (Irwin, 2009). The result also shows that the anaemia was regenerative. The infected untreated group had the lowest RBC count, but within the normal range of 5.5 - 8.5 $x10^{12}$/L (Nalubamba *et al.,* 2015), apparently due to haemoconcentration as a result of dehydration, thus masking the anaemia (Zygner *et al.,* 2007). This also confirms the findings of Torbica *et al.* (2013) that RBC count in dogs with babesiosis normalises 15 days post-inoculation. The result obtained in the infected + imidocarb group suggested the babesiosis was an uncomplicated type which agrees with the finding of Torbica *et al.* (2013), who reported rapid recovery after imidocarb administration in canine babesiosis. The infected + imidocarb + ALA dogs had the highest RBC count, which may be attributed to the antiprotozoal, anti-inflammatory and antioxidant effects exerted by a combination of imidocarb dipropionate and ALA.

In the present study, a decreased Hb concentration below the normal range of 12 - 18 g/dL (Nalubamba *et al.,* 2015) in the infected untreated dogs at day 11 PI, compared to the uninfected untreated dogs revealed extravascular haemolysis caused by *B. canis vogeli*, but without evidence of haemoglobinaemia. This result agrees with the extravascular haemolysis obtained by Salem and Farag (2014), in canine babesiosis. This may cause pale mucous membrane or icterus and tissue hypoxia because the RBCs lack haemoglobin; a molecule which contains haem, the oxygen-transporting protein in the RBC (Gupta and

David, 2014), responsible for the reddish colouration of the blood. Haemoglobin is a large globulin protein that is composed of two alpha- and two beta- chains, which form a four-iron-containing haeme group, binding to four globulin chains. The Hb concentrations in the infected groups compared to the uninfected untreated dogs at day 8 PT revealed an improvement in the treated groups.

The innate immunity in the infected untreated dogs plays a major role in the protection against babesiosis, which results into the generation of natural killer cells (NKC) and macrophages, which help in erythrophagocytosis of the parasitised RBC. However, the low Hb concentration as a result of dehydration in the infected + ALA dogs, which fell below the normal range, may be attributed to erythrophagocytosis of parasitised RBCs (Crnogaj *et al.*, 2017). The decreased Hb concentrations in the infected untreated and infected + imidocarb and infected + ALA groups at day 15 PT, compared to the uninfected untreated group revealed an on-going extravascular haemolysis. The haemolysis may be associated with parasitaemia in the infected untreated group and relapse in the infected + imidocarb group. On the contrary, the normal Hb concentrations in the infected + imidocarb + ALA dogs recorded might be due to the antioxidant and anti-inflammatory effects exerted by ALA (Tatar *et al.*, 2016). Gomes and Negrato (2014) reported that ALA is a mitochondrial antioxidant that regenerates vitamins C and E, and glutathione. It exhibits antioxidant activities in cell membranes and cytosol (Golbidi *et al.*, 2011; Gomes and Negrato, 2014). Therefore, co-administration of ALA with heavy metals maintains and normalises haematological changes especially in arsenic toxicity (Nikolic *et al.*, 2015), which, in addition, may be reason for the normal Hb concentration recorded in the infected + imidocarb + ALA dogs. The normal Hb concentrations obtained at day 43 PT in all the infected groups (infected untreated, infected + imidocarb, infected + ALA and infected +

imidocarb + ALA), similar to that of the uninfected untreated dogs indicated an improvent in the health status of the dogs. The finding may be due to either haemoconcentration in the infected untreated and infected + ALA dogs or the ameliorative effect exerted by a combination of imidocarb dipropionate and ALA in the treatment of canine babesiosis. The combination yielded the highest Hb concentration which may be the result of their synergistic effect in the amelioration of erythroperoxidation that usually occurs in canine babesiosis (Uchendu *et al.*, 2014).

The increase in size of the RBC, though within the normal range of 64 - 73 fl (Eichenberger *et al.*, 2016), in the infected untreated and infected + ALA dogs at base-line and day 8 PT, compared to PT days 15 and 43 indicated a normocytic anaemia and the presence of reticulocytes in circulation. Spherocytes which are small spherical RBCs that are two-third of the diameter of the normal RBC, have decreased surface area compared to their volume and are densely haemoglobinised. Spherocytosis occurs when there is destruction of the erythrocyte membrane as reported in canine babesiosis (Reddy *et al.*, 2016), anti-erythrocyte antibodies attached to the RBCs and phagocytised by macrophages in the spleen and liver. The macrophages only phagocytise part of the RBC membrane; thus creating spherocytes, which, when in large quantity is pathognomonic of immune-mediated haemolytic anaemia (Piek, 2011). Torbica *et al.* (2013) reported spherocytosis as a normal occurrence in regenerative haemolytic anaemia.

The decreased size of RBC obtained in the infected + imidocarb dogs at day 15 PT compared to the base-line and day 8 PT; and at day 43 PT compared to day 8 PT indicated microcytosis, which may be due to iron deficiency observed in the advanced stage of canine babesiosis The iron-deficiency is sequel to chronic blood loss. Iron becomes

deficient for erythropoiesis, which is an essential component of the haeme group of haemoglobin. Therefore, in the absence of iron, haemoglobin cannot be produced; and adversely affects enzyme activity which impairs receptor expression in the RBCs and subsequently its release into the circulation. This has been reported by various authors (Niwetpathomwat *et al.,* 2006; Esmaeilnejad *et al.,* 2012; Ajith *et al.,* 2017; Bilwal *et al.,* 2017) in canine babesiosis.

The decreased MCV in the infected + ALA dogs at PT days 15 and 43, respectively compared to the values obtained at base-line and day 8 PT indicated reduction in volume of the RBCs, either due to haemolysis (extravascular) or partial erythrophagocytosis. The finding has been reported by Zvorc *et al.* (2010) who recorded a decrease in MCV in dogs infected with *B. c. canis* before and after treatment with imidocarb dipropionate. Similarly, the decrease in size in the infected + imidocarb + ALA dogs, though within the normal range of 64 - 73 fl (Eichenberger *et al.,* 2016) was an evidence of microcytosis, which may be with anaemia. The lower MCV at day 11 PI in the infected + ALA dogs compared to that of the uninfected untreated dogs, though within the normal range of 64 - 73 fl (Eichenberger *et al.,* 2016) was indicative of normocytic anaemia. The result was apparently associated with the release of inflammatory cytokines that suppress erythropoiesis and sequestration of iron via increasing hepcidin. However, at day 8 PT, the slightly increased MCV in the infected + ALA dogs, though lower than that in the infected untreated and infected + imidocarb dogs, may suggest that ALA alone may not abolish or prevent haemolysis. The normal volume recorded in the infected untreated may be caused by dehydration, while in the infected + imidocarb may be as a result of restoration of the RBCs to the normal MCV value after administration of imidocarb dipropionate. The MCV was slightly elevated at day 43 PT in the infected untreated, infected + imidocarb and

infected + imidocarb + ALA dogs and slightly lower MCV in infected + ALA dogs, although all the values were within the normal range, indicating normocytic anaemia.

The increased MCHC at day 11 PI compared to the base-line in the infected untreated dogs indicates normocytic normochromic anaemia. This was because the value fell within the normal range of 32.0 - 36.3 g/dL (Salem and Farag, 2014). On the contrary, at day 8 PT, MCHC decreased below the normal range, compared to the base-line and day 11 PI, suggesting normocytic hypochromic anaemia. The finding may be due to the decrease in the ratio of the weight of haemoglobin to the volume of the RBC. This simply implies that the individual RBC contained less haemoglobin concentration due to iron deficiency. This may be as a result of diminished iron absorption or excessive iron loss, which is often observed in canine babesiosis. The MCHC obtained at PT days 15 and 43 compared to the base-line, and at days 11 PI and 8 PT were all within the normal range, indicated normocytic normochronic anaemia. The result agrees with the findings of previous authors (Fabisiak *et al.,* 2010; Torbica *et al.,* 2013), who reported normocytic normochromic anaemia at the onset of babesiosis in dogs. Pavel and Malanchus (2013) also reported normocytic normochromic anaemia as one of the different types of anaemia found in horses infected with babesiosis. The decreased MCHC at day 8 PT in the infected + imidocarb dogs below the normal range compared to day 11 PI indicated normocytic hypochromic anaemia. Similar result was obtained in the infected + imidocarb + ALA dogs at day 8 PT compared to day 11 PI. This finding confirms the result of the study conducted by Simoes *et al.* (2011), who reported normocytic hypochromic anaemia in a German shepherd bitch infected with *Babesia microti*-like *spp.* in Northern Portugal. The normocytic hypochromic anaemia recorded in the present study later developed to normocytic normochromic anaemia (Sikorski *et al.,* 2010; Shah *et al.,* 2011; Eichenberger *et al.,* 2016) at PT days 15

and 43; which also confirms the findings of Furlanello *et al.* (2005), who reported normocytic normochromic anaemia in 74% dogs with babesiosis. Koster *et al.* (2015) however, reported normocytic normochromic regenerative anaemia at the onset of *Babesia* infection. Normocytic normochromic anaemia was observed in all the groups during the present study; except at day 8 PT when normocytic hypochromic anaemia was recorded in the infected untreated, infected + imidocarb, and infected + imidocarb + ALA groups, respectively.

In the present study, the WBC count was within the normal range of 6 - 17 x 10^3/µL (Eichenberger *et al.,* 2016) which agrees with the findings of Eichenberger *et al.* (2016) who reported that all survivors of *B. canis* infection have WBC counts within the normal range compared to the non-survivors. The result agrees with the finding of Adebayo *et al.* (2016), who reported an insignificant difference in WBC counts between *Babesia*-positive and -negative dogs. The slight decrease in WBC in all the infected untreated, infected + imidocarb and infected + imidocarb + ALA groups at day 11 PI may be attributed to the ability of WBCs to bind with intracellular adhesion molecule (ICAM-1) via receptors (Chakravorty and Craig, 2005; Hildebrand *et al.*, 2005). The ICAM-1 may contribute to the initial decrease in WBC count observed at the onset of babesiosis and mediate WBC trans-endothelial migration, which is a component of a cascade of molecular interactions (Rafaj *et al.*, 2013). It may also be due to the ability of platelets to bind with activated extracellular cells, which interact with WBCs and induce secondary capture. The binding leads to neutrophil-endothelial interaction which could contribute to the initial decrease in WBC count before treatment, triggering vascular inflammation (Mine *et al.*, 2001). The slight increase recorded at day 8 PT compared to day 11 PI confirms the findings of Kucer *et al.* (2008) who reported no significant ($P > 0.05$) difference in WBC counts between

complicated and uncomplicated babesiosis before and on day 2 PT, but increased subsequently at day 7 PT. At day 15 PT, there was a decrease in the WBC count in all the infected groups compared to that of day 8 PT; except in the infected + imidocarb + ALA dogs, where an increase was recorded. Subsequently, the WBC count further decreased in the infected + imidocarb, infected + ALA and infected + imidocarb + ALA groups. The result of the WBC count obtained in the present study showed that the normal WBC count may not be a consistent prognostic marker of survival in canine babesiosis.

All the neutrophil counts obtained in the infected groups and at different durations fell below the the normal range of 3 - 11.5 $x10^3$/µL (Eichenberger *et al.,* 2016), indicating neutropenia (Wormser *et al.,* 2015). The neutropenia may be as a result of increased migration of neutrophils into the tissues (Zamokas *et al.,* 2014) as a response to inflammatory cytokines and chemokines, leading to decreased bone marrow storage. It may also be due to immune-mediated neutropenia in haemophagocytic syndrome, which occurs in canine babesiosis (Kirtz *et al.,* 2012; Koster *et al.,* 2015). This corroborates the finding of Reddy *et al.* (2016), who reported neutropenia in *Babesia*-infected dogs in India.

The result of the lymphocyte counts in the infected groups throughout the study showed that the counts were all above the normal range of 1.0 - 4.8 $x10^3$/µL (Salem and Farag, 2014), indicating lymphocytosis; apparently due to increased production of antibodies against *Babesia* parasites during infection, and attributed to chronic syndrome of anaemia and fever. Lymphocytosis has been reported by Al-Saad (2009), Salem and Farag (2014) and Reddy *et al.,* 2016 in canine babesiosis.

The elevated NLR in all the infected groups at day 11 PI compared to the uninfected untreated group was an evidence of inflammation and oxidative stress due to canine babesiosis. The result demonstrated that NLR may serve as an indicator of inflammatory reaction and prognosis of diseases, as suggested by Zhang *et al.* (2014). The NLR plays a major role in systemic inflammatory response syndrome (SIRS), observed in complicated babesiosis (Omobowale *et al.,* 2017). The NLR is used to differentiate between complicated and uncomplicated babesiosis (Kucer *et al.,* 2008; Omobowale *et al.,* 2017), and a prognostic marker of oxidative stress in gastric cancer patients (Zhang *et al.,* 2014). The slightly decreased NLR in the infected untreated, infected + imidocarb and infected + ALA dogs at day 8 PT, compared to the uninfected untreated group indicated a good prognosis after the different treatments. The slightly elevated NLR in the infected + imidocarb + ALA dogs may indicate neutrophil-dependent inflammatory response and a decreased lymphocyte-mediated response (Zhang *et al.,* 2014). The result confirms the finding of Li *et al.* (2014), that ALA exhibits both antioxidant and immunoprotective activities (Kim *et al.,* 2014; Li *et al.,* 2014).

The decreased platelet counts observed in all the infected dogs at day 11 PI compared to the base-line data and the uninfected untreated group indicated marked immune-mediated thrombocytopenia. The thrombocytopenia may be attributed to immune-mediated attack against megakaryocytes in the bone marrow, thus, leading to thrombocytopenia (Singh *et al.,* 2014). It may also be due to immune-mediated clearance/destruction (Narurkar*et al.,* 2017) or apoptosis of platelets during haemolytic anaemia in babesiosis (Salem and Farag, 2014; Koster *et al.,* 2015; Solano-Gallego *et al.,* 2016). The thrombocytopenia may also be related to sequestration of platelets in the spleen, which corroborates the splenomegaly that was observed, and the damaged platelets may also be sequestrated in the liver and lungs

(Kubo *et al.*, 1998; Minemura *et al.*, 2009). It may also occur due to adherence of platelets to RBC membranes (Goddard *et al.*, 2015). The persistent increase in platelet counts at PT days 8 and 15, compared to day 11 PI in all the infected groups, suggests a reactive thrombocytosis, which is a common finding in immune-mediated haemolytic anaemia, chronic inflammatory diseases and iron deficiency anaemia. It may be attributed to the release of inflammatory cytokines including IL-6, which stimulates thrombopoietin secretion and megakaryopoiesis and, subsequently, production of platelets (Machlus *et al.*, 2016). Furthermore, it may also suggest marked thrombocytosis, observed in early stage of recovery from immune-mediated thrombocytopenia, and it is usually transient (Swann and Skelly, 2016). The marked decrease in platelet counts in all the infected dogs at day 43 PT, compared to day 15 PT, is suggestive of platelets sequestration in the spleen and liver (Kubo *et al.*, 1998).

In the present study, the glucose concentrations in all the groups were within the normal range of 3.3 - 5.5 mmol/L (Defauw *et al.*, 2012), indicating normoglycaemia. The result agrees with the findings of Jacobson (2006), who recorded normoglycaemia in dogs infected with babesiosis. Hypoglycaemia is a common complication of canine babesiosis (Keller *et al.*, 2004; Matijatko *et al.*, 2009; Reddy *et al.*, 2014), which may be caused by increased glucose consumption by the parasite, resulting in hypermetabolism and anaerobic glycolysis by the parasites (Keller *et al.*, 2004). It is often associated with severe anaemia, icterus and vomiting, especially in puppies, and may be misdiagnosed as cerebral babesiosis because of the observed frequent neurologic signs. The normoglycaemia recorded in this study might be caused by the *B. canis vogeli*, which has a low virulence and uncapable of inducing severe clinical manifestations. The ameliorative role of ALA as a metabolic antioxidant and its co-administration with imidocarb dipropionate at day 15 PT

may be responsible for the decreased glucose concentration. This result agrees with the finding of Ghelani *et al.* (2017), that supplementation with ALA suppresses hyperglycaemia in rats.

The RBCs are sensitive to oxidative injury induced by increased generation of ROS that occurs during babesiosis. This is because they are constantly exposure to high oxygen tension and elevated PUFA in their membranes and also their lack of nucleus and organelles (Eze *et al.*, 2017). At day 11 PI, the high percentage haemolysis recorded at 0.3% NaCl concentration in all the infected groups indicated lipoperoxidation of the PUFA in the RBC membrane caused by *Babesia canis vogeli*. The increased haemolysis may be due to the increased glycosylation of the RBC membrane protein or an alteration of the Na^+/K^+-ATPase in the RBC membrane (Eze *et al.*, 2017). The result agrees with the findings of Shah *et al.* (2011), that parasitaemia increases RBC osmotic fragility. The increased fragility may be due to an immune-mediated haemolytic anaemia, as previously observed by Paes *et al.* (2013). The highest percentage haemolysis recorded in the infected untreated group at day 8 PT was an evidence that RBC was sensitive to the hypotonic solution at 0.7% and 0.5% NaCl concentrations, compared to the uninfected untreated, infected + imidocarb, infected + ALA and infected + imidocarb + ALA, respectively. This result indicated that the infected untreated group had the highest percentage of haemolysis and was highly unstable at 0.7% NaCl concentration. It may be related to ROS-induced haemolysis, occurring during babesiosis, causing increased lysis and lipid peroxidation of the RBC membrane, thus rendering the cell highly susceptible to the osmotic pressure. The infected + imidocarb + ALA dogs had the lowest percentage haemolysis at 0.5% NaCl concentration, indicating that the RBCs were relatively stable and, consequently, had minimal haemolysis, compared to other infected groups. The result may be attributed to the

ameliorative properties of ALA as an anti-inflammatory and antioxidant that prevents oxidative injury of membranes and, thus, maintains the RBC integrity (Zavareh *et al.,* 2015; Ahmet *et al.,* 2016; Hwang *et al.,* 2016; Yang *et al.,* 2016). The infected untreated group at day 15 PT had the highest percentage haemolysis at 0.9%, 0.7% and 0.5% NaCl concentrations compared to the remaining groups. The finding indicated that the RBC were highly permeable and had a low tensile strength, thus their high susceptibility to hypotonic lysis. On the contrary, the infected + imidocarb + ALA had the lowest percentage haemolysis at 0.5% and 0.3% NaCl concentrations respectively, compared to the other infected groups. This result may be attributed to the ameliorative effect exerted by the co-administration of imidocarb and ALA in the treatment of canine babesiosis. It may also be due to the amphiphilic nature of ALA to exhibit its antioxidant activity in the membrane and also in the cytosol (Gomes and Negrato, 2014). It has been shown that ALA also has the ability to cross blood-brain barrier to maintain the integrity of many cells during exposure to oxidative stress (Shay *et al.,* 2009). Similar antioxidant effect of ALA was obtained at day 43 PT in the infected + imidocarb + ALA group at 0.5% NaCl concentration, and apparently indicating the ability of ALA to regenerate other natural antioxidants in the body, including vitamins C and E and glutathione. The result agrees with the finding of Lektib *et al.* (2016), who reported that vitamin E is regenerated by ALA, and that ALA protected the RBC membrane from oxidative stress, improved membrane fluidity and reduced haemolysis that occured during oxidative injury of RBC. ALA has been reported to diminish the effect of iron-induced ROS on RBC membrane integrity which decrease osmotic fragility and reduce the rate of haemolysis (Ali *et al.,* 2015).

All the serum potassium concentrations obtained in this study indicated hypokalaemia. Hypokalaemia is an uncommon finding in malaria and babesiosis, associated with decreased potassium intake and respiratory alkalosis. It may be an indicator of early renal tubular dysfunction or as a response to alkalosis. The finding agrees with the result obtained by Lobetti and Jacobson (2001), that hypokalaemia occurs in dogs with babesiosis-related renal dysfunction. Increased serum aldosterone concentration in some cases of canine babesiosis may be responsible for the hypokalaemia (Adaszek *et al.,* 2012; Zygner *et al.,* 2012; Gojska-Zygner and Zygner, 2014). ALA has been reported to protect against potassium cyanide-induced seizures, which may be due to nitric oxide over-production, and maintenance of intracellular antioxidant defence mechanism (Abdel-Zaher *et al.,* 2011).

The decreased serum calcium concentration recorded in all the infected groups at day 11 PI indicated mild hypocalcaemia, which was consistent from PT days 8 to 15. This finding may be attributed to increased binding of calcium to protein, especially albumin, abnormal functioning of the parathyroid glands, decreased absorption and excessive loss of Ca^{2+}. The decreased calcium binding may be induced by increase in muscle and neural excitability. In dogs, the decreased Ca^{2+} is responsible for clinical manifestations including muscle tremors, convulsion and ataxia. It may also be attributed to the weakness and the hyperphosphataemia observed in this study. Hypocalcaemia has been reported previously in canine babesiosis (Eichenberger *et al.,* 2016). However, at day 43 PT, the Ca^{+} concentration normalised, indicating an improvement. This agrees with the finding of Qingwu *et al.* (2007), who reported that treatment with ALA increased intracellular Ca^{2+} concentration in myotubes. Hypocalcaemia has been reported to occur in malaria (Prabha *et al.,* 1998; Ayoola *et al.,* 2005; Rani *et al.,* 2015). This may be due to Ca^{2+} concentration in the

internal compartment for metabolism (Gazarini *et al.*, 2003), deficiency in Ca^{2+} during digestive and renal complications. *Babesia canis vogeli* may increase the cell permeability to Ca^{2+} (Tiffert *et al.,* 2000). Infected cells increase Ca^{2+} permeability, which leads to reduction of blood Ca^{2+} in malaria patients. Thus, the *Babesia* parasites may adhere to glomerular capillaries causing renal insufficiency and, leading to increased urinary excretion of calcium, which results in hypocalcaemia (Rani *et al.,* 2015). Calcium plays a major role in cellular death and membrane damage. Calcium ions' entry and deposition in mitochondria causes uncoupling of phosphorylation and extensive mitochondrial damage, preventing the cell from regenerating energy, and reversal of the damage (Jacobson and Clark, 1994).

The elevated serum phosphate recorded at days 11 PI and 8 PT in all the infected groups above the normal range of 3.3 - 5.5 mg/dL (Cortadellas *et al.,* 2010) indicates mild hyperphosphataemia. The hyperphosphataemia may be associated with metabolic acidosis characterised by tissue hypoxia, which is a common sequel in canine babesiosis. It may be caused by dehydration, hypoparathyroidism (due to increased renal reabsorption), chronic renal diseases (occurring due to decreased excretion of phosphorus), and decreased glomerular filtration rate in babesiosis (Camacho *et al.,* 2004; Solano-Gallego *et al.*, 2016). Normal serum phosphate concentration was recorded in all the treated groups at days 15 PT and 43 PT, respectively because of the effects of the treatments.

Magnesium is a major intracellular cation that serves as a catalyst or activator in many enzymatic reactions. It is needed for all the reactions that involve ATP, including Mg-ATP complex, which is the immediate substrate in these reactions. The majority of the body's magnesium is present in the bone (50%), skeletal muscle and soft tissue, while only

roughly 1% is present in the blood; thus, the level measured in serum or plasma may be a poor indicator of total body stores. In this study, the serum magnesium concentrations of the infected groups at days 11 PI and at PT days 8, 15 and 43, compared to the uninfected untreated group were not significant, though they were above the normal range of 1.89 - 2.50 µg/dL (Martin *et al.,* 1994). The increased concentration of serum magnesium in all the infected groups compared to the unifected untreated groups at day 11 PI, may explain the haemolysis or red blood cell burst that occurs in canine babesiosis. This agrees with the findings of Koseoglu *et al.* (2010) that haemolysis interfers with routine biochemical parameters in humans. Subsequently, persistent decrease in serum Mg concentration recorded in all the infected groups at days PT 8 to 15 compared to day 11 PI, though higher than the normal range, may be an indication of hypocalcaemia reported in this study. It may be attributed to the ability of magnesium to inhibit the parathyroid hormone or stimulate calcium uptake into the bones. The hypermagnesaemia recorded in all the infected groups at day 43 PT may be associated with decreased excretion, resulting from reduced glomerular filtration.

All the serum creatinine concentrations were within the normal range of 44 - 133 (Defauw *et al.,* 2012), except the decreased creatinine concentration obtained in the infected untreated and infected + imidocarb groups at day 11 PI, which may indicate a loss of muscle mass due to anorexia. The result agrees with the finding of anorexia and weight loss in this study. Similarly, decreased serum creatinine concentration recorded in the infected + imidocarb group at day 8 PT may be attributed to increased glomerular filtration rate and decreased creatinine production in the liver. It may also occur as a result of the effect of babesiosis on the kidneys; and worsened by the administration of imidocarb dipropionate

which has been attributed to degeneration of the kidneys and causing nephrotoxicity (Dyachenko e*t al.,* 2012).

The elevated BUN concentration at day 11 PI in all the infected groups compared to the base-line and the uninfected untreated group, though within the normal range of 7 - 27 mg/dL in dogs (Bradea *et al.,* 2014), may be attributed to the build-up of ammonia as a result of haemolysis that occurs in canine babesiosis (Tantary *et al.,* 2014). De Scally *et al.* (2004) reported that elevation of BUN and creatinine concentrations has been ascribed to a loss of 60 - 75% loss of nephron function, which was not investigated in this study and is further confirmed by the lack of azotaemia in this study. Subsequently, at PT days 8 and 15 respectively, there was a marked decrease in the BUN concentration in all the infected groups, signifying liver dysfunction or starvation. An elevation of BUN alone is unreliable for diagnosis of renal insufficiency in babesiosis; because the elevation may be due to catabolism of lysed erythrocytes (Jacobson and Clark, 1994).

In all the infected groups, elevated bilirubin beyond the normal range of dogs 1.71 - 8.55 µmol/L (Mizukami *et al.,* 2012) was recorded at day 11 PI indicating severe hyperbilirubinaemia. The result may be attributed to the rapid breakdown of RBCs during babesiosis or obstruction of the bile duct and gall bladder obstructions and liver disease. The mild haemolysis recorded, was not severe enough to cause jaundice. Several authors (Torbica *et al.,* 2013; Vishnurahav *et al.,* 2014) have reported hyperbilirubinaemia in babesiosis. At day 8 PT, there was a decrease in the level of bilirubin, which may be attributed to the reduced haemolysis as a result of the attempt by the dogs to combat the infection in the infected untreated dogs, and the effects of the different therapeutic interventions. Marked bilirubin elevation was recorded in the infected untreated group at

day 15 PT, compared to the slight increase in the infected + imidocarb, infected + ALA, and infected + imidocarb + ALA groups, which may be associated with the different degrees of haemolysis, and not necessarily due to hepatic dysfunction (Matijatko *et al.*, 2010). The decrease in bilirubin concentration at day 43 PT showed that there was no relapse, which may be due to the ameliorative role of ALA in the prevention of oxidative injury in RBCs as recorded in the infected + ALA and infected + imidocarb + ALA groups.

Normal CK concentration was recorded in all the groups at different durations, except at day 11 PI in the infected + imidocarb + ALA that had elevated CK activity above the normal range of 40 - 254 µ/L, apparently due to muscle injury as a result of the oxidative stress associated with babesiosis. Similarly at day 15 PT, the mild elevated CK activity in the infected untreated and infected + ALA groups may be confirmed by the myalgia recorded in this study. Creatine kinase catalyses the transport of phosphate to adenosine diphosphate in order to produce adenosine triphosphate (ATP), which is a major source of energy to tissues (Hamoda *et al.*, 2014). This result agrees with findings of Beck *et al.* (2017), who reported elevated creatine kinase activity in dogs infected with babesiosis.

The elevated serum total protein above the normal range of 5.5 - 7.5 g/dL (Gonde *et al.*, 2016) in all the infected untreated groups at days 11 PI, PT 8 and 43, indicated hyperproteinaemia. In the present study, dry muzzle and sunken eyeballs which are signs of dehydration were recorded and may be the reason for the hyperproteinaemia. Hyperproteinaemia has been reported in horses with babesiosis, and it is characterised by dehydration and inflammation (Kumar *et al.*, 2002; Barrera *et al.*, 2010). This finding may be suggestive of liver and kidney dysfunction and an increase in globulin concentration in response to the parasitic antigen, and the release of haemoglobin from the damaged RBCs

(Esmaeilnejad *et al.*, 2012). At days 8 PT mild hyperproteinaemia was recorded, but it re-occurred at day 15 PT in the infected untreated, infected + imidocarb and infected + imidocarb + ALA. Subsequently, at day 43 PT, elevated serum total protein (hyperproteinaemia) was recorded, which confirms the study by Esmaeilnejad *et al.* (2014), who reported a significantly ($P < 0.05$) higher serum total protein in sheep infected with *Babesia ovis*, compared to healthy group. Camacho *et al.* (2005) reported a significantly ($P < 0.05$) higher serum total protein in dogs infected with *Babesia annae*. The hyperproteinaemia recorded in the present study disagrees with the finding of Sivajothi *et al.* (2014), who reported decreased serum total protein and that of Shah *et al.* (2011), who did not obtain any significant alteration in total protein serum concentration in dogs infected with babesiosis.

The slight decrease in albumin concentration obtained at day 11 PI in the infected untreated, infected + ALA and infected + imidocarb + ALA groups below the normal range of 3.4 - 4.3 g/dL (Behling-Kelly, 2014), indicates hypoalbuminaemia. This may indicate dysfunction in the liver, kidneys, and the onset of an infection. It may also be linked to the hypocalcaemia that was recorded in this study, which agrees with the findings of previous authors (Solano-Gallego and Baneth, 2011; Solango-Gallego *et al.*, 2016) that hypoalbuminaemia occurs in dogs with babesiosis. At day 8 PT, all the infected groups showed severe hypoalbuminaemia, except the infected + imidocarb + ALA group that had slightly low serum albumin concentration, which demonstrated the ability of ALA to bind with albumin to prevent glycation (Kawabata and Packer, 1994). The remaining serum albumin concentrations in all the infected groups at day 15 PT were within the normal range except in the infected + imidocarb which showed hypoalbuminaemia. Similarly, the infected untreated and infected + ALA dogs demonstrated hypoalbuminaemia; and the

infected + imidocarb and infected + imidocarb + ALA groups, showed hyperalbuminaemia at day 43 PT, indicating dehydration.

The elevated serum globulin concentration in all the infected groups at day 11 PI, compared to the uninfected untreated and the normal range of 2.1 - 4.1 g/dL (Sikorski *et al.,* 2010), indicated severe hyperglobulinaemia. The hyperglobulinaemia reduced significantly at day 8 PT, but the value was still above the normal range, suggesting mild hyperglobulinaemia. The result may be attributed to overwhelming inflammation and potential chronic infection. It may also be a reflection of tissue damage caused by tissue hypoxia that is often associated with canine babesiosis. Hyperglobulinaemia has been reported in babesiosis (Esmaeilnejad *et al.,* 2012; Solano-Gallego *et al.,* 2016). The significant decrease in serum globulin concentration at day 15 PT in the infected untreated, infected + imidocarb and infected + imidocarb + ALA groups compared to the uninfected untreated group indicated hypoglobulinaemia, and the finding may be an indication of hepatic or renal dysfunction or an immune deficiency. However, the hyperglobulinaemia recorded at day 43 PT in all the infected groups compared to the uninfected and untreated group suggests that the dogs in the infected untreated group exhibited the chronic form of canine babesiosis.

The low A/G ratio in all the infected groups at days 11 PI, 8 PT and 43 PT, compared to the normal range of 0.7 - 1.0 (Sikorski *et al.,* 2010) indicated a compensatory response to hypoalbuminaemia or hyperglobulinaemia; recorded in this study. The increased A/G ratio obtained at day 15 PT in all the infected groups was also a compensatory response to probably the hypoglobulinaemia recorded in this study.

The elevated ALT activity at day 11 PI in the infected groups compared to the uninfected untreated in this study, though within the normal range of 8.2 - 57 U/L (Gonde *et al.*, 2016), indicated lack of its leakage from the liver due to hepatobiliary membrane damage, also lack of evidence of active hepatocellular injury and inflammation. The result agrees with the finding of Shah *et al.* (2011), who reported unaltered ALT activity in dogs infected with *Babesia canis*. The decreased ALT activity at day 8 PT in all the infected groups may be attributed to the effect of treatment in the oxidative damage of the liver. The result obtained at day 43 PT in the infected + imidocarb + ALA dogs may be attributed to the protective role of ALA as a cytoprotective antioxidant and its beneficial role in liver regeneration (Bustamante *et al.*, 1998; Dulundu *et al.*, 2007; Hussein *et al.*, 2015).

All the AST activities recorded in this study were within the normal range of 16 - 43 U/L (Vojta *et al.*, 2012) and they agree with the findings of Shah *et al.* (2011) that normal AST activities occur in dogs infected with *Babesia canis*. The slight increase in AST activity in the infected + ALA group at PT days 8 and 15, may be attributed to mild hepatic injury, resulting in AST release from the mitochondria and the RBCs that occurs during haemolysis, thus causing an elevated serum AST activity (Ellinger *et al.*, 2011). The result agrees with the finding of Zygner *et al.* (2011), who demonstrated increased AST activity in dogs with babesiosis. The reduced AST activity in the infected + imidocarb + ALA group may be associated with the protective role of ALA in hepatic condition, as reported by Yang *et al.* (2014) in a case of hepatic steatosis.

At day 11 PI, the elevated serum ALP activity above normal range of 10 - 75 U/L (Vojta *et al.*, 2012) in the infected + ALA, and infected + imidocarb + ALA groups may be related to the mild anaemia, observed in the groups, apparently inducing hypoxia and hypoxic liver

damage that usually occur in canine babesiosis (Zygner *et al.,* 2011). The finding may also be attributed to the increased corticosteroid ALP isoenzyme that is produced in the liver and localised on the hepatocyte plasma membrane lining the bile canaliculi and sinusoids. It is usually observed in chronic illnesses, possibly secondary to stress and concomitant increase in endogeneous glucocorticoid secretion, and observed with a concurrent increase in serum GGT activity, specific for liver dysfunction. The marked elevation in serum ALP activity in all the infected groups at PT days 8, 15 and 43, compared to the uninfected untreated group may indicate cholestasis that may be observed in canine babesiosis.

In the present study, there was a marked increase in serum GGT activity above the normal range of 1 - 5 U/L (Vojta *et al.,* 2012) at day 11 PI in the infected untreated and infected + imidocarb. This finding may be attributed to decreased uptake and excretion of bilirubin and cholestasis, which may occur in canine babesiosis. This is confirmed by the concurrent increase in ALP activity and the hyperbilirubinaemia, indicating liver disease probably caused by *B. canis vogeli*. This agrees with the finding of Crnogaj *et al.* (2010) who reported increased GGT activity in dogs with the complicated form of *B. canis* infection. However, the infected + ALA, and infected + imidocarb + ALA groups had normal serum GGT activity which subsequently increased by day 8 PT. There was a decrease in serum GGT activity in all the infected groups at day 15 PT, except in the infected untreated dogs which may be attributed to the administration of imidocarb dipropionate and ALA in the treated groups, and the presence of *B. c. vogeli* in the infected untreated group. Subsequently, all the infected groups normalised by day 43 PT, suggesting the effectiveness of the different therapies.

The lowered serum copper concentration at day 11 PI compared to base-line in the infected untreated and infected + imidocarb + ALA group may be related to the mild anaemia observed in canine babesiosis. Copper is important for the production of haemoglobin and RBC, and ceruloplasmin is the main copper-containing blood plasma protein that oxidises Fe (II) ferroxidase activity. It mediates iron efflux from cells and also neutralises ROS (Ramos *et al.,* 2016). There was an increase in the infected + imidocarb and infected + ALA groups at day 11 PI compared to base-line Cu^{2+} concentration. The result agrees with the findings of Seyrek *et al.* (2009), who reported an increase in plasma copper concentrations in infectious processes, resulting in an elevated serum Cu^{2+} concentration (Pasa *et al.,* 2003; Seyrek *et al.,* 2009). At day 8 PT, the lowered serum Cu^{2+} concentration in the infected untreated, infected + imidocarb and infected + ALA groups compared to the uninfected untreated indicated anaemia and oxidative stress in the groups. The result agrees with the findings of Chaudhuri *et al.* (2008), who reported the importance of lowered level of blood iron in oxidative stress, Cu and Zn in the pathogenesis of anaemia and oxidative stress in *B. gibsoni*-infected dogs. However, this is contrary to the increased serum Cu^{2+} in the infected + imidocarb + ALA dogs at day 11 PI compared to PT days 8 and 15, which may be related to the normal PCV value recorded in this group. It may be attributed to the anti-inflammatory and antioxidant role of ALA and imidocarb in the prevention of haemolysis resulting in the lowered serum Cu^{2+} in the other infected groups. The reduced serum Cu concentration in the infected + ALA dogs may be attributed to the role of ALA as a therapeutic metal-chelating antioxidant (Ou *et al.,* 1995; Gomes and Negrato, 2014). ALA chelates divalent transient metal ions; both *in vivo* and *in vitro*, but by different mechanisms of action (Smith *et al.,* 2004). It chelates Cu^{2+}, Mn^{2+}, Pb^{2+} and Zn^{2+}, while the reduced form chelates Hg^{2+} and Fe^{2+}. Both Fe^{2+} and Cu^{2+} are mediators of ROS (Gomes and Negrato, 2014). Serum Cu^{2+} concentration was normalised, at day 43 PT in all the infected groups

compared to the uninfected untreated group may be related to the normal PCV value, and RBC counts, recorded in the dogs, thus resulting in the rise in serum Cu^{2+} concentration.

Zinc is a mineral that acts as a component or activator of numerous metalloenzymes and hormones (Spodniewska *et al.*, 2012). It is an essential component of the antioxidant defence against *Babesia*-induced ROS. It is utilised in the synthesis of antioxidant enzymes (Esmaeilnejad *et al.*, 2014). The slightly elevated serum Zn recorded in the infected untreated, infected + imidocarb, and infected + ALA groups at day 11 PI, compared to base-line and the uninfected untreated group, may be attributed to its antioxidant role and an immune response to canine babesiosis. It may also indicate the onset of an early stage of babesiosis. This is contrary to the findings of Esmaeilnejad *et al.* (2014), who reported a decreased serum-Zn in sheep infected with *Babesia ovis*. The decreased serum Zn concentration in the infected + imidocarb + ALA group may be attributed to the ameliorative role of the co-administration of imidocarb and ALA in prevention of ROS damage. The elevated serum Zn recorded in the infected + imidocarb, infected + ALA, and infected + imidocarb + ALA groups at day 8 PT, compared to day 11 PI may be due to the effects of treatment in the different groups.

The increase in serum iron in the infected untreated, infected + imidocarb, infected + ALA and infected + imidocarb + ALA groups at day 11 PI compared to both the base-line and the uninfected untreated group may be attributed to the haemolytic anaemia that was observed at day 11 PI, occurring due to destruction of RBCs and excessive RBC turn-over in the peripheral blood. It confirms the finding of El-Far *et al.* (2014), who demonstrated an increased iron concentration in calves infected with *Babesia bigemina*. The result disagrees with that of Salem and El-Sherif (2015), who reported a decrease in iron in horses infected

with *Theileria equi*. However, there was a decrease in serum iron in the infected + ALA group compared to the base-line, which may be attributed to copper deficiency or excess zinc. Similar finding was obtained by Hamoda *et al.* (2014) who reported a decreased iron in cattle infected with *B. bigemina*. The decreased serum iron in the infected untreated, infected +imidocarb and infected + ALA dogs at day 8 PT, compared to day 11 PI suggests sequestration of iron within the cells, including the macrophages and hepatocytes in canine babesiosis. The result of this study confirms the finding of Furlanello *et al.* (2005), who reported a decrease in total iron concentration in the serum of dogs infected naturally with large *Babesia spp*. However, in the result obtained in the infected + imidocarb + ALA dogs may be as a result of generation of inflammatory cytokine-induced synthesis of ferritin and hepcidin, resulting in sequestration of iron within macrophages. At day 15 PT, the elevated serum iron concentration in the infected untreated, infected + imidocarb and infected + ALA groups may be as a result of release of iron from intracellular stores into circulation. On the contrary, the decrease in serum iron concentration in the infected + imidocarb + ALA dogs may be attributed to antioxidant and metal-chelating activities of ALA (Gomes and Negrato, 2014). At day 43 PT, all the infected groups had normal serum iron, compared to the uninfected untreated indicating a positive response to the treatment, and also corroborates the normal PCV and RBC obtained in this study.

The increased MDA concentration in the RBC in all the infected groups at day 11 PI, compared to the uninfected untreated group suggests oxidative stress of RBCs caused by lipid peroxidation which may account for the anaemia recorded in this study. The result of the present study further showed that oxidative stress plays a role in the pathogenesis of anaemia in canine babesiosis, and it agrees with the finding of Crnogaj *et al.* (2015), that RBC MDA concentration rose on the first day of presentation of dogs infected with *B.*

canis canis. The higher MDA concentration in the RBC at day 8 PT compared to that of day 11 PI, in the infected untreated, infected + imidocarb and infected + imidocarb + ALA dogs may be attributed to the persistent increase in MDA concentration due to slow recovery of the dogs from the deleterious effects of increasing lipid peroxidation (Zvorc *et al.*, 2010; Crnogaj *et al.*, 2015; Solano-Gallego *et al.*, 2016). This was particularly true of the infected untreated group, indicative of the extent of the RBC damage. On the contrary, the decreased MDA concentration in the RBC, recorded in the infected + ALA dogs may be associated with the administration of ALA, demonstrated to improve endothelium dysfunction by Xiang *et al.* (2010). The antioxidant and anti-inflammatory properties of ALA may contribute to its mitigating effect against lipid peroxidation in the infected + ALA dogs. Subsequently, the persistent increased MDA concentration in the RBC at PT days 15 and 43 in all the infected groups, except the infected + imidocarb + ALA at day 15PT, suggests an increased RBC oxidation, which may not only be due to oxidation of the parasitised RBCs, but also non-parasitised RBCs. This result corroborates the finding of Radwan *et al.* (2013), who reported decreased RBC MDA concentration in *Babesia*-infected calves, treated with a combination of imidocarb + vitamin E. The result confirms the findings of Murase *et al.* (1995), who obtained oxidation of non-parasitised RBCs in *B. gibsoni* infection. It also agrees with the finding of Crnogaj *et al.* (2017) that MDA concentration increases in *Babesia*-infected dogs with complicated and uncomplicated forms of babesiosis, mild and moderate anaemia compared to the control group. The decrease in MDA concentration in RBCs in the infected + imidocarb + ALA dogs at day 15 PT may be attributed to the ameliorative effect of the co-administration of imidocarb and ALA on RBCs.

Platelet contains GPx which is important in immune system and blood clotting mechanisms of the body. Marked reduction in the platelet GPx activity occurred at day 11 PI in all the infected groups, suggest lipid peroxidation of the platelets and may account for the thrombocytopenia recorded in this study. It may also be attributed to the antioxidant effect of GPx activity in scavenging ROS (hydroperoxide and lipid peroxide), generated in canine babesiosis, thus leading to its reduction in circulation. The result agrees with the finding of Salem *et al.* (2016), who demonstrated the role of GPx activity in catabolism and prevention of ROS effect in *Babesia*-infected calves. Selenium deficiency may also be a contributing factor, a major componenet of platelets which combines with sulphur-containing amino acids (cysteine and methionine) to form glutathione peroxidase. The result of the study is in agreement with the finding of Yasini *et al.* (2013), who obtained a decrease in GPx activity between days 23 - 38 post-infection in anaplasmosis in sheep. The decreased GPx activity in dogs with moderate anaemia, compared to those with mild anaemia in babesiosis is indicative of oxidative stress and its severity (Crnogaj *et al.*, 2015). The result agrees with the finding of Araujo *et al.* (2008), that decreased platelet GPx activity occur in humans with malaria, which may be due to production of GPx activity in response to ROS by *Plasmodium spp*. The increased platelet GPx activity at day 8 PT in all the infected groups suggests reduced lipoperoxidation in the platelets. The increase in platelet GPx activity in the present study may be attributed to the ability of the imidocarb dipropionate and ALA to ameliorate platelet lipid peroxidation and sequestration, recorded at day 11 PI; this results explains the elevated thrombocytes at day 8 PT. The highest platelet GPx activity recorded at day 8 PT in the infected + imidocarb dogs among the infected groups may be attributed to the ability of imidocarb dipropionate to eliminate large *Babesia spp.* within 24 h of administration (Brandao *et al.*, 2003). The persistent, decreased platelet GPx activity at PT days 15 and 43 in the infected untreated and infected + ALA

dogs may be suggestive of an overwhelming generation of ROS, contrary to the increased platelet GPx activity in the infected + imidocarb dogs. This finding suggests minimal oxidation of platelets in the infected + imidocarb dogs.

The SOD is an antioxidant enzyme produced in the body to neutralise a specific oxygen-derived free radical called superoxide. The serum SOD in all the infected groups at day 11 PI compared to base-line indicated an increase in production of SOD to scavenge superoxide (O_2^-) radical, generated post-infection, which will further be converted to molecular oxygen and hydrogen peroxide. Subsequently, the persistent decrease in serum SOD activity at day 15 PT, compared to day 8 PT may be indicative of scavenging of free superoxide radicals from circulation, resulting in reduction of antioxidant biomarker in both the treated and untreated groups, and in scavenging of ROS, evidenced by the decrease in cell SOD activity that was recorded. The result agrees with the finding of Salem *et al.* (2016), who reported the antioxidant role of SOD in protection of RBC against oxidative damage. The increase in serum SOD activity in all the infected dogs at day 43 PT may be attributed to the antioxidant property of SOD activity to reduce/eliminate oxidative stress. It agrees with the findings of Radwan *et al.* (2013), who reported an increase in SOD activity in *Babesia*-infected calves treated with a combination of imidocarb and vitamin E.

Tumour necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine that plays a significant role in the pathogenesis of babesiosis and malaria, and influences the severity of the diseases (Hemmer *et al.*,2000; Krause *et al.*,2007; Zygner *et al.*, 2014). Complications of canine babesiosis, such as multiple organ dysfunction syndrome and hypotension, may result from the parasite, the host or parasite-host interactions (Matijatko *et al.*,2012). The marked increase in TNF-α (pro-inflammatory) in all the infected groups at day 11 PI,

compared to the base-line and the uninfected untreated group may indicate that inflammation is involved in the pathogenesis of canine babesiosis. It may be attributed to activation of the immune response and parasitaemia control, as related to infection caused by haemoparasite such as babesiosis (Paim *et al.*, 2013). The increase may also be associated with sequestration of parasitised RBCs in babesiosis, resulting in localised release of cytokines and more severe adverse effects on organs in which sequestration occurs (Jacobson and Clark, 1994). At day 8 PT, a significant ($P > 0.05$) increase in TNF-α in infected untreated and infected + ALA compared to day 11 PI and the uninfected untreated group suggests that TNF-α may play a role in pathogenesis and immune response to diseases (Faria *et al.*, 2011). However, the lowest TNF-α concentration recorded in the infected + imidocarb + ALA dogs may be attributed to the anti-inflammatory role of ALA in inhibiting the production and expression of cytokines and membrane co-stimulatory molecules (Maczurek *et al.*, 2008). Subsequent increase recorded in the infected + imidocarb and infected + ALA groups may be associated with the inflammation that occurs in canine babesiosis and induction of the effector molecules of systemic inflammatory response syndrome. Cytokines form a complex network with a high level of synergism, redundancy and duplication (Jacobson and Clark, 1994). They are important in host defence against blood parasites and excessive or imbalance production may be detrimental to the host. TNF-α is an acute phase protein that induces a cytokine cascade and increases vascular permeability accompanied by macrophage and neutrophil recruitment to the infection site. TNF-α may cause clinical aggravation due to its pro-inflammatory activity and enhances the protective effect of IFN-γ in the hosts (Beineke *et al.*, 2008).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

In conclusion, the study showed that:

i. molecular identification and characterisation of the isolate confirmed the presence of *Babesia canis vogeli* in Zaria, and PCR detected infection in babesiosis with low parasitaemia which has been hitherto difficult by microscopy. The isolate had 100% identity with *Babesia canis vogeli* from India, Brazil and from a cat in Taiwan.

ii. *Babesia canis vogeli* infection caused mild alterations in clinical manifestations, including fever and lymphnode enlargement at day 3 PI; bilateral ocular discharge and pale ocular mucous membrane at day 6 PI; and inappetence, dullness, lethargy, myalgia, sunken eyeballs, weight loss and splenomegaly at day 12 PI. Treatment with imidocarb dipropionate and ALA alleviated the clinical manifestations of dry muzzle, ocular discharge, myalgia and splenomegaly.

iii. the rectal temperature and pulse rate reduced ($P < 0.05$) in the infected + imidocarb + ALA dogs at PT days 15 (37.87 ± 0.16°C; 77.33 ± 7.16 beats/min, respectively) and 43 (37.61 ± 0.13°C; 72.13 ± 4.04 beats/min, respectively).

iv. decreased PCV was recorded in the infected untreated dogs (39.62 ± 4.39%, 33.12 ± 3.33% and 32.08 ± 1.40%, pre-inoculation, 11 PI and 15 PT), infected + imidocarb dogs (38.47 ± 6.89%, 32.20 ± 1.01% and 29.70 ± 1.99%, pre-inoculation, 11 PI and 15 PT), infected + ALA dogs (41.02 ± 2.82%, 31.98 ± 3.04% and 34.64 ± 4.25%, pre-inoculation, 11 PI and 15 PT) except in the infected + imidocarb + ALA dogs (45.20 ± 3.76%, 33.00 ± 4.05% and 47.40 ± 3.72%, pre-inoculation, 11 PI and 15 PT), at day 15 PT. Infected + imidocarb + ALA dogs had the highest erythrocyte count at PT days 15 (6.10 ± 0.44 x 10^{12}/L) and 43 (6.99 ± 0.56% x 10^{12}/L), compared to infected untreated (4.68 ± 0.21

$x10^{12}$/L and 5.55 ± 0.16 $x10^{12}$/L, 15 PT and 43 PT), infected + imidocarb (4.36 ± 0.34 $x10^{12}$/L and 6.45 ± 0.63 x 10^{12}/L, 15 PT and 43 PT) and infected + ALA (5.28 ± 0.63 x 10^{12}/L and 6.41 ± 0.37 x 10^{12}/L, 15PT and 43 PT) dogs, respectively. At days 11 PI and 15 PT, the Hb concentration decreased in the infected untreated (10.90 ± 1.07 g/dL and 10.80 ± 0.49 g/dL), infected + imidocarb (11.77 ± 1.02 g/dL and 10.31 ± 0.56 g/dL), infected + ALA (10.87 ± 1.14 g/dL) groups, respectively except in the infected + imidocarb + ALA group at day 15 PT (13.80 ± 0.91 g/dL).

v. marked thrombocytopenia was recorded in all the infected groups at day 11 PI.

vi. high percentage haemolysis was obtained at 0.5% NaCl concentration in all the infected groups at day 11 PI. Infected untreated had the highest haemolysis (51.71 ± 0.38%, 81.14 ± 4.34%, 86.31 ± 1.39% and 68.76 ± 5.25%, pre-inoculation, 8 PT, 15 PT and 43 PT, respectively), while infected + imidocarb + ALA had the lowest percentage haemolysis (53.75 ± 0.57%, 56.45 ± 0.80%, 34.93 ± 0.37% and 56.39 ± 2.36%, pre-inoculation, 8 PT, 15 PT and 43 PT, respectively).

vii. erythrocyte malondialdehyde concentration was significantly ($P < 0.05$) higher at pre-inoculation, days 11 PI, PT days 8, 15 and 43, in the infected untreated group (0.84 ± 0.29 µM, 2.13 ± 0.23 µM, 2.59 ± 0.14 µM, 3.00 ± 0.42 µM and 6.68 ± 0.22 µM), infected + imidocarb (0.84 ± 0.09 µM, 1.72 ± 0.21 µM, 2.43 ± 0.09 µM, 3.09 ± 0.89 µM and 6.55 ± 0.96 µM), infected + ALA (0.55 ± 0.26 µM, 2.52 ± 0.34 µM, 2.11 ± 0.25 µM, 2.30 ± 0.30 µM and 8.61 ± 1.47 µM) and infected + imidocarb + ALA (0.53 ± 0.32 µM, 2.13 ± 0.24 µM, 2.53 ± 0.30 µM, 2.14 ± 0.33 µM and 7.74 ± 1.54 µM), respectively.

viii. serum superoxide dismutase activity was significantly ($P < 0.05$) lower in the infected untreated group (1.97 ± 0.18 U/L and 1.08 ± 0.10 U/L, pre-inoculation and 8 PT), infected + imidocarb (2.12 ± 0.29 U/L and 1.10 ± 0.15 U/L, pre-inoculation and 8 PT), infected + ALA (2.14 ± 0.20 U/L and 1.19 ± 0.06 U/L, pre-inoculation and 8 PT) and infected +

imidocarb + ALA (2.14 ± 0.39 U/L and 0.95 ± 0.95 U/L, pre-inoculation and 8 PT) groups, respectively. Lowest SOD activities were recorded in the infected untreated at PT days 15 (0.33 ± 0.05 UL) and 43 (1.83 ± 0.11 UL).

ix. platelet glutathione peroxidase activity significantly ($P < 0.05$) decreased in the infected untreated group (117.94 ± 8.17 U/L, 6.73 ± 1.31 U/L, 22.50 ± 3.44 U/L and 10.18 ± 2.25 U/L, pre-inoculation, days 11 PI, 15 PT and 43 PT, respectively), infected + imidocarb (104.73 ± 23.27 U/L, 9.46 ± 2.73 U/L, 9.67 ± 1.68 U/L and 24.60 ± 4.42 U/L, pre-inoculation, days 11 PI, 15 PT and 43 PT, respectively), infected + ALA (67.58 ± 6.18 U/L, 11.16 ± 2.86 U/L, 21.17 ± 2.04 U/L and 10.52 ± 1.11 U/L, pre-inoculation, days 11 PI, 15 PT and 43 PT, respectively) and infected + imidocarb + ALA (121.55 ± 0.84 U/L, 5.61 ± 0.78 U/L, 13.46 ± 2.11 U/L and 13.25 ± 1.47 U/L, pre-inoculation, days 11 PI, 15 PT and 43 PT, respectively) except at day 8 PT (63.96 ± 0.53 U/L, 72.90 ± 1.34 U/L, 51.92 ± 6.24 U/L and 60.99 ± 3.63 U/L, infected untreated, infected + imidocarb, infected + ALA and infected + imidocarb + ALA, respectively).

x. A significant ($P < 0.05$) increase in tumour necrosis factor-alpha concentration was obtained in the infected untreated group (97.48 ± 51.69 pg/mL, 200.92 ± 72.00 pg/mL, 276.21 ± 70.76 pg/mL and 157.83 ± 41.90 pg/mL, pre-inoculation, days 11 PI, 8 PT and 15 PT, respectively), infected + imidocarb (48.44 ± 11.17 pg/mL, 230.31 ± 21.61 pg/mL, 230.19 ± 70.56 pg/mL and 272.74 ± 73.48 pg/mL, pre-inoculation, days 11 PI, 8 PT and 15 PT, respectively), infected + ALA (127.02 ± 47.19 pg/mL, 113.37 ± 44.32 pg/mL, 168.06 ± 31.50 pg/mL and 183.92 ± 75.59 pg/mL, pre-inoculation, days 11 PI, 8 PT and 15 PT, respectively) and infected + imidocarb + ALA (148.46 ± 27.19 pg/mL, 180.46 ± 79.02 pg/mL, 172.53 ± 10.15 pg/mL and 121.11 ± 89.08 pg/mL, pre-inoculation, days 11 PI, 8 PT and 15 PT, respectively).

xi. Alpha-lipoic acid exerted some synergistic and regenerative effects following co-administration with imidocarb dipropionate in amelioration of *Babesia*-induced haemolytic anaemia, thrombocytopenia and oxidative stress associated with canine babesiosis.

6.2 Recommendations

6.2.1 Specific recommendations

i. Diagnosis of *Babesia spp.* should include molecular identification and characterisation, especially in cases of low parasitaemia where microscopy is unreliable.

ii. Supplementation with alpha-lipoic acid should be encouraged for clinicians and dog owners due to its protective and ameliorative roles on blood cells, tissues and organs in canine babesiosis.

iii. Co-administration of imidocarb dipropionate and alpha-lipoic acid in the treatment of canine babesiosis should be advocated to clinicians.

iv. Other pro-inflammatory cytokines, including IL-1, IL-6 and IFN-γ should be investigated to further elucidate the pathophysiology of canine babesiosis.

v. The duration for the study of relapse should be extended more than a month.

6.2.2 General recommendations

i. Prevention and control of ticks, the vector that transmits babesiosis, should be encouraged.

ii. Routine haematological examination should be carried out in dogs suspected for babesiosis on presentation.

6.3 Contributions to Knowledge

i. *Babesia canis vogeli* caused alterations in clinical manifestations and vital parameters of Nigerian indigenous dogs, alleviated with the combination of imidocarb dipropionate and ALA, better than imidocarb dipropionate alone.

ii. *Babesia canis vogeli* induced haematological changes including erythrocyte haemolysis, were ameliorated with the co-administration of imidocarb dipropionate and ALA, better than imidocarb dipropionate alone.

iii. *Babesia canis vogeli* induced changes in serum proteins, liver function enzymes and trace elements in Nigerian indigenous dogs were ameliorated by the synergistic effects of imidocarb dipropionate and ALA, better than imidocarb dipropionate alone.

iv. *Babesia canis vogeli* increased erythrocyte malondialdehyde concentration, and activities of platelet glutathione peroxidase and serum superoxide dismutase in Nigerian indigenous dogs, ameliorated by the combination of imidocarb dipropionate and ALA, better than imidocarb dipropionate alone.

v. *Babesia canis vogeli* infection increased in tumour necrosis factor-alpha concentration in Nigerian indigenous dogs which was reversed by the combination of imidocarb dipropionate and ALA, better than imidocarb dipropionate alone.

6.4 Limitations of the Study

i. Difficulty in identification of *Babesia canis vogeli* in Nigerian indigenous dogs microscopically.

ii. Inability to acquire a *Babesia canis vogeli*-positive donor dog.

iii. Inadequate funding.

iv. Shortage of dogs.

v. Unstable power supply for storage of samples and analyses.

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APPENDICES

APPENDIX I: *Babesia canis vogeli* forward

TATGTACTGTTTCCGTAGGTGAACCTGCGGAAGGATCATTCACATTGATGGTAC
CTAGCACTTGTGCTTTCGCGGGACGCGCTGTCGAGGTTGTCCTGGGCGGCGGTC
TCGTCTGGCAACGGAGCCGGCGACTCGCCGGTTCTACCCTGTTGGGCGTTGCCC
CCTCTCCACCCCACTGGGGCGTTGCGTAAGCTCTGCCCCGGGTTAGGCCCCGAG
CGTGGATTCCGTTGGTTTCCGGGCTTGGGGATGTGGCGCGCCTGGGCGCGTCAG
GGTCTTTGACCTCCGCGGCTTGTCCGATTTGCAACTCCGCTTGACTGTGTCGAGT
ATTGAAATTCTAAACTTTCAGCGATGGATGTCTTGGCTCACACAACGATGAAGG
ACGCAGCGAATTGCGATACGCATTATGACTTGCAGACTTCTGCGATTTAACAGA
CCTCCGAACGTAACCAACACACCGCCTCTGCTCGCACGCGGTACTCCCGTTTCA
GTGAGCCCCCTTTTCTGAGGCCTTGCCTTTTTGGCAGCCTTGCGAGTGGGTGGTT
GTGCCGCCCGTCTGGGGGAGAGAGAGATGTGTAAGATTAAAGGAAGGAGAAG
TCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTCACATTGATGGT
ACCTAGCACTTGTGCTTTCGCGGGACGCGCTGTCGAGGTTGTCCTGGGCGGCGG
TCTCGTCTGGCAACGGAGCCGGCGACTCGCCGGTTCTACCCTGTTGGGCGTTGC
CCCCTCTCCACCCCACTGGGGCGTTGCGTAAGCTCTGCCCCGGGTTAGGCCCCG
AGCGTGGATTCCGTTGGTTTCCGGGCTTGGGGATGTGGCGCGCCTGGGCGCGTC
AGGGTCTTTGACCTCCGCGGCTTGTCCGATTTGCAACTCCGCTTGACTGTGTCG
AGTATTGAAATTCTAAACTTTCAGCGATGGATGTCTTGGCTCACACAACGATGA
AGGACGCAGCGAATTGCGATACGCATTATGACTTGCAGACTTCTGCGATTTAAC
AGACCTCCGAACGTAACCAACACACCGCCTCTGCTCGCACGCGGTACTCCCGTT
TCAGTGAGCCCCCTTTTCTGAGGCCTTGCCTTTTTGGCAGCCTTGCGAGTGGGT
GGTTGTGCCGCCCGATTGCGTGGAGGAGTTGGCTGGCCAAGGTCGTGGCTGTG
GTTCTACTCTGTGGAGCTGCGGCCATTTGCCTCGAAGCATTTTGTGTATGTGAT
ACCGAATCTTCTGCGAGGCTGTTGGGCTCTGCCCAAGACTGCGGTGAGCCGTCT
GACTCCGTGTCATCTATTCCGCTCCTGAAATCGGGTGAGGCTATCCGCTGAATT
TAAGCATATAATTAAGCGGCAGAAAAGAAAATAACCATGATTCCCTCGTGAAC
CTTATCACTTAAAGGAAGGAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTG
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GCCGGTTCTACCCTGTTGGGCGTTGCCCCCTCTCCACCCCACTGGGGCGTTGCG
TAAGCTCTGCCCCGGGTTAGGCCCCGAGCGTGGATTCCGTTGGTTTCCGGGCTT
GGGGATGTGGCGCGCCTGGGCGCGTCAGGGTCTTTGACCTCCGCGGCTTGTCCG
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GGATGTCTTGGCTCACACAACGATGAAGGACGCAGCGAATTGCGATACGCATT
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CCTTTTTGGCAGCCTTGCGAGTGGGTGGTTGTGCCGCCCGATTGCGTGGAGGAG
TTGGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTCACATTGATGGTACCTAG
CACTTGTGCTTTCGCGGGACGCGCTGTCGAGGTTGTCCTGGGCGGCGGTCTCGT
CTGGCAACGGAGCCGGCGACTCGCCGGTTCTACCCTGTTGGGCGTTGCCCCCTC
TCCACCCCACTGGGGCGTTGCGTAAGCTCTGCCCCGGGTTAGGCCCCGAGCGTG
GATTCCGTTGGTTTCCGGGCTTGGGGATGTGGCGCGCCTGGGCGCGTCAGGGTC
TTTGACCTCCGCGGCTTGTCCGATTTGCAACTCCGCTTGACTGTGTCGAGTATTG
AAATTCTAAACTTTCAGCGATGGATGTCTTGGCTCACACAACGATGAAGGACG
CAGCGAATTGCGATACGCATTATGACTTGCAGACTTCTGCGATTTAACAGACCT
CCGAACGTAACCAACACACCGCCTCTGCTCGCACGCGGTACTCCCGTTTCAGTG

AGCCCCCTTTTCTGAGGCCTTGCCTTTTTGGCAGCCTTGCGAGTGGGTGGTTGTG
CCGCCCGATTGCGTGGAGGAGTTGGCTGGCCAAGGTCGTGGCTGTGGTTCTACT
CTGTGGAGCTGCGGCCATTTGCCTCGAAGCATTTTGTGTATGTGATACCGAATC
TTCTGCGAGGCTGTTGGGCTCTGCCCAAGACTGCGGTGAGCCGTCTGACTCCGT
GTCATCTATTCCGCTCCTGAAATCGGGTGAGGCTATCCGCTGAA

APPENDIX II: ***Babesia canis vogeli*** **reverse**

ACGAACATCGGCAGGCTGCCAAAAGGCAAGGCCTCAGAAAAGGGGGCTCACT
GAAACGGGAGTACCGCGTGCGAGCAGAGGCGGTGTGTTGGTTACGTTCGGAGG
TCTGTTAAATCGCAGAAGTCTGCAAGTCATAATGCGTATCGCAATTCGCTGCGT
CCTTCATCGTTGTGTGAGCCAAGACATCCATCGCTGAAAGTTTAGAATTTCAAT
ACTCGACACAGTCAAGCGGAGTTGCAAATCGGACAAGCCGCGGAGGTCAAAG
ACCCTGACGCGCCCAGGCGCGCCACATCCCCAAGCCCGGAAACCAACGGAATC
CACGCTCGGGGCCTAACCCGGGGCAGAGCTTACGCAACGCCCCAGTGGGGTGG
AGAGGGGGCAACGCCCAACAGGGTAGAACCGGCGAGTCGCCGGCTCCGTTGCC
AGACGAGACCGCCGCCCAGGACAACCTCGACAGCGCGTCCCGCGAAAGCACA
AGTGCTAGGTACCATCAATGTGAATGATCCTTCCGCAGGTTCACCTACGGAAAC
CTTGTTACGACTTCTCCTTCCTTTAAGTGAAAAAGGGTTCACA