COMPARATIVE EVALUATION OF PARASITOLOGICAL AND SEROLOGICAL METHODS FOR THE DETECTION OF NATURAL *EHRLICHIA CANIS* INFECTION IN DOGS IN ZARIA METROPOLIS, KADUNA STATE, NIGERIA

BY

BILKISU UMARU-SULE

DEPARTMENT OF VETERINARY PARASITOLOGY AND ENTOMOLOGY,

FACULTY OF VETERINARY MEDICINE,

AHMADU BELLO UNIVERSITY,

ZARIA

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BY

Bilkisu UMARU-SULE DVM (ABU) 2006

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DEPARTMENT OF VETERINARY PARASITOLOGY AND ENTOMOLOGY

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APRIL, 2018

DECLARATION

I declare that the work in this dissertation entitled **'Comparative Evaluation of Parasitological and Serological Methods for The Detection of Natural *Ehrlichia canis* Infection in Dogs in Zaria Metropolis, Kaduna State, Nigeria**' has been carried out by me in the Department of Veterinary Parasitology and Entomology under the supervisions of **Prof. O.O. Okubanjo**, **Prof. B.D.J.George** and **Prof. D.A.Y. Adawa**. 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/diploma at this or any other institution.

Bilkisu UMARU-SULE _____ _____

Signature Date

CERTIFICATION

This dissertation entitled "**COMPARATIVE EVALUATION OF PARASITOLOGICAL AND SEROLOGICAL METHODS FOR THE DETECTION OF NATURAL *EHRLICHIA CANIS* INFECTION IN DOGS IN ZARIA METROPOLIS, KADUNA STATE, NIGERIA**" by Bilkisu UMARU-SULE, meets the regulations governing the award of Master of Science Degree of the Ahmadu Bello University, Zaria and is approved for its contribution to Knowledge and Literary presentation.

Prof. O.O. Okubanjo
Chairman, Supervisory Committee _____ _____

Signature Date

Prof. B.D.J. George
Member, Supervisory Committee _____ _____

Signature Date

Prof. D.A.Y. Adawa
Member, Supervisory Committee _____ _____

Signature Date

Prof. O.O. Okubanjo
Head, Department of Veterinary Parasitology and
Entomology, Faculty of Veterinary Medicine,
A.B.U. Zaria _____ _____

Signature Date

Prof. Sadiq .Z. Abubakar
Dean, School of Postgraduate Studies,
Ahmadu Bello University, Zaria _____ _____

Signature Date

DEDICATION

This work is dedicated to Allah (SWT), The Enabler and Knower of All Things.

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ABSTRACT

Canine ehrlichiosis caused by *Ehrlichia canis* is an important and potentially fatal disease of dogs. It has been reported worldwide and is transmitted by the dog brown tick *Rhipicephalussanguineus*. Detection of *E. canis* infection can be done by direct identification of inclusion bodies or morulae of *E. canis* in leucocytes from smears made from blood or from buffy coat and lymphnode aspiration by immunofluorescent antibody test (IFAT) and Dot-ELISA. Molecular techniques such as Polymerase chain reaction (PCR) are also used as diagnostic methods. This research work was undertaken to compare the sensitivity of four different diagnostic tests for *Ehrlichia* viz: thin blood and buffy coat smear examinations, *Ehrlichia* culture test and a commercially available rapid immunochromatographic assay (Rapid *Ehrlichia canis* antibody) test in the detection of natural *E. canis* infection in dogs in relation to breed, age and sex. A total number of 100 dogs comprising 62 males, 38 females were randomly selected under natural conditions from residential areas of Zaria, Kaduna state; 77 of the dogs were local, 12 local-exotic crosses and 11 exotic with age range between 1-3 years being the majority (58%). The intracytoplasmic ehrlichial organisms were observed in buffy coat smears of 2 dogs (2.0%) while *Ehrlichia* culture detected 20 cases (20.0%). Rapid *Ehrlichia canis* antibody test detected 83 infected dogs (83.0%). The two buffy coat positive were also positive for the *Ehrlichia* culture and rapid *E. canis* antibody tests. Dogarawa ward had a high prevalence rate of 40.0% using the *Ehrlichia* culture test, while Basawa revealed a 90.0% rate using the rapid antibody test. There was no significant statistical association between buffy coat smears and the breed, age or sex ($p>0.05$). A significant statistical association was observed between the rapid antibody test with the breed ($p=0.019$) and age ($p=0.001$) while sex showed no significant association ($p>0.05$). No significant association was observed for age, sex and breed in relation to *Ehrlichia* culture test. There was a slight level of agreement between the applied detection

methods (buffy coat smear vs *Ehrlichia* culture; 0.151; p< 0.04, buffy coat smear vs serology; 0.008; p< 0.518, serology and *Ehrlichia* culture test; 0.069; p< 0.110). Dogs recruited for the study were apparently healthy. However, physical examination revealed fever in 15% of the dogs, 15% had ticks, 10% were emaciated and had rough hair coats, 3% had pale mucus membranes, 1% had diarrhea, blood tinged urine, scrotal edema, and worms in faeces while 2% were anorexic. Thrombocytopenia was the most consistent haematological finding in both the positive and negative groups of dogs showing also a significant statistical difference, followed by leukocytosis (lymphocytosis). Serum Biochemical analysis revealed increased albumin levels in the infected groups of dogs followed by a decrease in urea in both the infected and uninfected groups. Hyperproteinaemia however, was the most consistent finding in both groups. The study showed that in addition to haematological characterization, a combination of parasitological and serological investigation methods is recommended for rapid detection of canine ehrlichiosis.

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CHAPTER ONE

INTRODUCTION

1.1 General Introduction

Dogs are susceptible to a number of Ehrlichial diseases currently recognized to be caused by *Ehrlichia canis, E. chaffeensis* and *E. ewingii* (Straube, 2010). Among them canine ehrlichiosis (CE) caused by *E. canis* is an important and potentially fatal disease of dogs. CE is an infectious disease caused by the gram negative bacterium *E. canis* (Ristic and Huxsoll, 1984) transmitted by the ubiquitous dog brown tick *Rhipicephalus sanguineus* (Lewis *et al.*, 1977). *E. canis* infection has been reported as a worldwide canine infectious disease (Ristic and Holland, 1993) being more common in tropical and subtropical regions (Neer and Harrus, 2006). *E.canis* infection has been reported in Nigeria (Trimnell *et al.,* 1989; Abdullahi *et al*., 1990).

The incubation period of *E. canis* infection can range from 8 to 20 days (Castro *et al*., 2004; Neer and Harrus, 2006) and the disease is characterized by fever, anorexia, depression, lymphadenomegaly, splenomegaly, vasculitis and ocular and musculoskeletal signs (Waner *et al*., 1999a; Dagnone *et al*., 2001; Castro *et al.,* 2004). Thrombocytopenia is the most common abnormality in naturally or experimentally infected dogs (Waner *et al*., 1995).

There are three phases of *E. canis* infection, each varying with severity. The acute phase, occurring several weeks after infection and lasting up to a month, can lead to fever and lowered peripheral blood cell counts due to bone marrow depression (Waner *et al*., 1997). The subclinical phase has a duration that varies from months to years (Codner and Farris-Smith, 1986; Neer and Harrus, 2006). During this phase the animal could show high anti-*E. canis*antibody titers (Waner *et al*., 1997), persistent thrombocytopenia, and leucopenia with no apparent clinical signs

(Codner and Farris-Smith, 1986; Waner *et al*., 1997; Harrus *et al*., 1998a, 2004). Severe pancytopenia, haemorrhagic diastasis, and organism debilitation can characterize the chronic phase (Neer and Harrus, 2006).

Diagnosis of *E. canis* infection can be done by direct identification of inclusion bodies or morulae of *E. canis* in leucocytes from smears made from blood (Elias, 1991) or from buffy coat and lymphnode aspiration by immunofluorescent antibody test (IFAT) and Dot-ELISA (Cadman *et al*., 1994; Nakaghi *et al*., 2008; Oliviera *et al*., 2000). Molecular techniques such as PCR (Wen *et al*., 1997; Nakaghi *et al*., 2008) are used as diagnostic methods.

1.2 Statement of Research Problems

Laboratory diagnosis of *E. canis* infection is a challenging task, since the disease presnts with varied clinical, haematological, as well as biochemical abnormalities (Waner, 2008). Ehrlichiosis in dogs is a fatal tick-borne disease caused by the obligate intracellular pathogen, *E. canis* (Greene and Harvey; 1990). Infected dogs manifest a wide range of clinical signs varying from mild weight loss to a severe and often fatal haemorrhagic syndrome (Dagnone *et al*., 2003; Machado, 2004; Nakaghi *et al*., 2008).

Ehrlichial infections can mimic several other diseases and non- specific clinical signs can make the diagnosis difficult (Cohn, 2003). Dogs in the acute phase of the disease show dramatic improvement in haematologic and clinical responses within 24-48 hours after therapy (Greene and Harvey, 1990). It is when the disease is not treated in the initial stages that it goes on to damage the systems of the host (Laksmanan *et al*., 2007). However, clinically healthy dogs are carriers of the organism in the sub-clinical phase of the disease, which increases the possibility of

transmission to other dogs (Laksmanan *et al*., 2007). Dogs in the chronic phase of the disease may not improve on treatment, thus leading to poor prognosis (Kuehn and Gaunt, 1985; Greene and Harvey, 1990). Hence diagnosis in the early stage of the infection is important to ensure successful treatments (Greene and Harvey, 1990).

Microscopic demonstration of intracytoplasmic organisms in infected monocytes is indicative of CE (Soulsby, 1986; Urquhart, 1987); but very few blood samples reveal the organism owing to low levels of parasitaemia. Cell culture isolation method is reported to be very sensitive and definitive; but not a convenient method as it requires several days to obtain results and thus defeats the whole purpose of rapid detection (Waner *et al*., 1999a). In Nigeria, it's intensive nature coupled with power outages also constitute a major obstacle. Serological methods such as Indirect Flourescent Antibody Test (IFAT) and Western Blot (WB) assay are effective for detection of antibodies to *E. canis* but failure of the techniques to differentiate ongoing and past infections, and early infections (1 to 3 weeks post infection), limit their reliability as confirmatory indicators of the disease (Iqbal *et al*., 1994). Polymerase Chain Reaction (PCR) based detection of *E.canis* has gained confidence within the scientific community but it requires sophisticated laboratories and accurate standardization. The test can be more expensive and generally takes time to be analyzed and the results can be subjectively interpreted.

1.3 Justification of the Study

Dogs are important household pets and most of the times kept for various other reasons which include security purposes, sheep herding and protection against predators, hunting and guide dogs leading the blind. There has been an increased interest in keeping dogs in Nigeria mainly as

pets or security dogs and, in some parts of the country for food (Kamani *et al*., 2011). In Zaria, dogs are kept mainly as household guards and are allowed to roam freely. A few of these dogs are restricted during the daytime. The roaming nature of dogs allows the spread of both vectors and disease agents thus, exposing them to parasitism, which is one of the most serious health hazards in canine practice and the affected dog may harbor parasites with zoonotic implications, thereby, constituting health hazards to their owners and the general public (Adejoke, 2005). A few decades ago, ehrlichioses were considered to only have veterinary relevance. The first human infection with *E. chaffeensis* was diagnosed in 1986 raising the awareness of *Ehrlichia* spp. as zoonotic pathogens (Maeda *et.al.*, 1987). *E. canis*, *E. chaffeensis*, and *E. ewingii* are all known to cause ehrlichiosis in humans (Straube, 2010).

Currently detection of canine ehrlichiosis is based on anamnesis, clinical presentations and confirmatory laboratory diagnosis. Microscopic demonstration of intracytoplasmic organisms in monocytes is indicative of the disease (Soulsby, 1986; Urquhart, 1987); but very few blood samples reveal the organism owing to low levels of parasitaemia. Cell culture isolation method is reported to be very sensitive and definitive; but not a convenient method as it requires several days to obtain results and thus defeats the whole purpose of early diagnosis (Waner *et al*., 1999a). Serological methods such as Indirect Flourescent Antibody Test (IFAT) and Western Blot (WB) assay are effective for detection of antibodies to *E.canis* but failure of the techniques to detect ongoing and past infections, and early infections (1 to 3 weeks post infection), limit their reliability as confirmatory indicators of the disease (Iqbal *et al*., 1994). Polymerase Chain Reaction (PCR) based detection of *E. canis* has gained confidence within the scientific community but it requires sophisticated laboratories and accurate standardization. The test can be more expensive and generally takes time to be analyzed. The present work aimed at comparing

the routine laboratory thin blood and buffy coat smears along with *Ehrlichia* culture test and a rapid serological test kit in the diagnosis of natural *E. canis* infection in dogs in Zaria. Haematological and biochemical alterations were evaluated to support clinical assessment of canine ehrlichiosis (since molecular and immunological methods are not readily available within the country).

1.4 Aim of the Study

To evaluate the most suitable technique of parasitological and serological procedures available for detecting natural *E.canis* infection in Zaria metropolis, Kaduna state.

1.5 Objectives of the Study

i. Identify the most suitable diagnostic technique for detection of *E.canis* (available to this work).

ii. To determine the prevalence of *E. canis* infection in dogs in Zaria metropolis**.**

iii. Evaluate the haematological and biochemical parameters associated with *E. canis* infection in the study area.

1.6 Research Questions

i. What is the most suitable detection technique for rapid diagnosis of *E. canis* infection to this study?

ii. Is *E. canis* present in the study area?

iii. What are the haematological and biochemical parameters associated with *E. canis* infection in the study area?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Introduction

Ehrlichiae are gram-negative, small, often pleomorphic, coccoid to ellipsoidal organisms that reside within cytoplasmic vacuoles either singly and more often in compact inclusions (morulae) present in mature or immature haematopoietic cells, especially mononuclear phagocytes such as monocytes and macrophages and for some species in myeloid cells such as neutrophils, in peripheral blood or in tissues, usually mononuclear phagocyte organs (spleen, liver, bone marrow, lymph node) of mammalian hosts (Ristic and Huxsoll, 1984). Ehrlichiae replicate in the membrane-bound vacuoles (parasitiphorous vacoules) in the cytoplasm of a specific type of host cells, chiefly monocytes or granulocytes. Ehrlichiae exist in nature as parasites of wild mammals, ticks and trematodes (Rikihisa, 1991). They replicate in the tick or trematode and are horizontally transmitted from infected cells in vectors to blood cells of animals and humans. Infection of blood cells of domestic and wild animals and humans with ehrlichial organisms may lead to a clinically apparent illness often accompanied with haematological abnormalities, lymphadenopathy, and increased liver enzyme activity (Dumler *et al*., 2001).

In the current amended taxonomy, Ehrlichia belongs to the kingdom Bacteria, phylum Proteobacteria, class α-proteobacteria, order Rickettsiales, family *Anaplasmataceae*, and genus *Ehrlichia* (Rikihisa, 1991). These gram- negative coccobacilli, are best known for their etiology in the transmission of a group of tick-borne illnesses known as Ehrlichiosis (Ismail *et al.*, 2011). They were originally classified according to the host cells and mammalian species they infected and their geographical location. Ehrlichiaewere initially grouped according to the type of blood cell most commonly infected (granulocyte, lymphocyte, monocyte, platelet), and disease classes

have been termed “granulocytic ehrlichiosis” or “monocytic ehrlichiosis”. However, this type of classification may be misleading because some of the *Ehrlichia sp* havebeen found in cells other than the chief target cell types. In addition, more than one species may be responsible for the broad category of “monocytic or granulocytic ehrlichiosis”. In the 1990s, the development of cell culture systems for most of these strictly intracellular organisms and advances in molecular biology techniques facilitated the phenotypic and genotypic characteristics of the Ehrlichias, and led to their phylogenetic positions being more clearly defined. The techniques have also greatly facilitated the diagnosis of ehrlichioses, and research on Ehrlichias has been stimulated by the findings of infections they cause in people (Buller *et.al.,* 1999).

Convincing phylogenetic data now shows that a series of significant flaws exists in the taxonomic structure of the families *Anaplasmataceae* and *Rickettsiaceae* in the order Rickettsiales (Rikihisa *et al*., 1992). It is now clear that a distinction between some members of the families *Rickettsiaceae* and *Anaplasmataceae* is not supported. Moreover, some members of the family *Anaplasmataceae*, the genera *Eperythrozoon* and *Haemobartonella*, are clearly not related to the genus *Anaplasma* and should be removed and reassigned within the family *Mycoplasmataceae* (Rikihisa *et al*., 1992). While no classification system fits all criteria perfectly, genetic data have become the objective standards and, when evaluated carefully, often closely predict similar biological and clinical behaviours (Dumler *et al*., 2001). Thus, the data compiled indicate that a sufficient genotypic and phenotypic relationship exists among the genera *Anaplasma*, *Cowdria*, *Wolbachia* and *Ehrlichia*, excluding *N. helminthoeca*, *E. sennetsu* and *E. risticii*, to merit unification into two separate genera (Dumler *et al*., 2001). Since the validly published names *Anaplasma* and *A. marginale* and *Ehrlichia* and *E. canis* predate *Cowdria* and *Wolbachia*, *Anaplasma* should be retained for the unified genus that encompasses the existing

Anaplasma species, the *E. phagocytophila* group, *E. bovis* and *E. platys*, while the genus *Ehrlichia* should be retained and used to describe members of the *E. canis* group, including *C. ruminantium*. This change further necessitates accommodation of the members of the *E. sennetsu* group within a single genus, *Neorickettsia*. Thus, a revised classification may be formulated that differentiates organisms in the order Rickettsiales into two families, *Rickettsiaceae*, which contains the rickettsiae (*Rickettsia, Orientia*) that occupy an intracytoplasmic compartment, and *Anaplasmataceae*, which contains the ehrlichiae (*Neorickettsia, Wolbachia, Ehrlichia, Anaplasma*) that occupy an intravacuolar compartment within infected host cells. Consequently, new combinations for the multiple genera and species that are involved must also be created. Weiss and Moulder, (1984) in the amended description of Rickettsiales (Gieszczykiewicz, 1939) proposed that the tribes *Rickettsieae, Ehrlichieae* and *Wolbachieae* should be abolished. Furthermore, all species formerly within the tribes *Ehrlichieae* and *Wolbachieae* are transferred into the family *Anaplasmataceae*. Weiss and Moulder, (1984) also proposed that the genera *Ehrlichia, Cowdria, Neorickettsia* and *Wolbachia* be transferred from the family *Rickettsiaceae* to the family *Anaplasmataceae*, a change that results in the elimination of all tribes within the family *Rickettsiaceae*. It was also proposed that the genera *Haemobartonella* and *Eperythrozoon* should be transferred from the family *Anaplasmataceae* to the order *Mycoplasmatales* and that *Coxiella, Rickettsiella, Francisella* (*Wolbachia*) *persica* and *Wolbachia melophagi* (Weisburg *etal*.,1989; Roux *et al*., 1997) should be removed from the family *Rickettsiaceae*.This proposal also requires amending of the description of the family *Rickettsiaceae* to specify that organisms infect host cells within the cytoplasm or nucleus and are not bounded by a vacuole. The family *Rickettsiaceae* includes only the genera *Rickettsia* and *Orientia* (Dumler *et al*., 2001). Ristic and Kreier (1984) proposed that the family *Anaplasmataceae* be amended to include species in the

genera *Wolbachia*, *Ehrlichia*, *Cowdria* and *Neorickettsia* and to retain species in the genera *Anaplasma* and *Aegyptianella*. This requires amendation of the description of the *Anaplasmataceae* to specify infection within a cytoplasmic vacuole of host cells that include erythrocytes, reticuloendothelial cells, bone marrow-derived phagocytic cells, endothelial cells and cells of insect, helminth and arthropod reproductive tissues (Dumler *et al*., 2001).

Currently, the genus *Ehrlichia* contains 5 recognized species: *E. canis, E. chaffeensis, E. ewingii, E. muris* and *E. ruminantium* (Centre for Food Security and Public Health, 2013). *E. canis* causes canine ehrlichiosis (CE). Canine ehrlichiosis did not gain meaningful attention until the 1970s when a significant number of cases were reported in German shepherds during the Vietnam War (Harrus and Waner, 2010). CE is also known as tropical canine pancytopenia, canine rickettsiosis, canine typhus, tracker dog disease or canine haemorrhagic fever (Centre for Food Security and Public Health, 2013).CE has since been reported in many parts of the world, mainly in the tropical and subtropical regions (apart from Australia and New Zealand) (Harrus *et.al*., 1997b). However, the geographical distribution of *E.canis* is expanding alongside that of its' main tick vector, the Brown Dog tick, *Rhipicephalus sanguineus* (Straube, 2010)

Co-infections of *Ehrlichia* with *Anaplasma, Rickettsia, Babesia* or *Bartonella spp* occur frequently as dogs are naturally exposed to multiple tick-borne pathogens (Straube, 2010). The awareness of co-infections is important in clinical practice, as diagnosis may be complicated by the presence of multiple pathogens.

Three different *Ehrlichia* species can cause canine ehrlichiosis*: E. canis, E. chaffeensis* and *E. ewingii* (Straube, 2010). All species can also affect humans. Clinical signs of the related canine

diseases (diseases like brucellosis, blastomycosis, Rocky Mountain spotted fever) in dogs are indistinguishable from those seen with CE.

A few decades ago, ehrlichioses were considered to only have veterinary relevance. The first human infection with *E. chaffeensis* was diagnosed in 1986 raising the awareness of *Ehrlichia spp*. as zoonotic pathogens (Maeda *et al*., 1987). Currently *E. canis*, *E. chaffeensis*, and *E. ewingii* are all known to cause ehrlichiosis in humans (Juliane, 2010).

2.2 *Ehrlichia canis*

The most recent description of *E.canis* is that of Ristic and Huxsoll (1984). Cells are Gram-negative, cocciod to ellipsoidal, often pleomorphic, intracytoplasmic bacteria that infect canid and perharps human cells of bone marrow derivation (Perez *et al*., 1996), predominantly cells in the monocytic lineage. The first microscopic observation of *Ehrlichia*-like intracellular organisms in mononuclear cells was described by M.-G.Kurloff at Paul Ehrlich's laboratory in 1889. This may be the reason why Moshkovsky in 1937 proposed the name genus *Ehrlichia* for this group of bacteria. *Rickettsia canis* (later renamed *Ehrlichia canis* by Moshkovsky) was the first *Ehrlichia* for which an association of *Ehrlichia sp*. with ticks and the clinical disease was made. In 1935, Donatien and Lestoquard described cocci in the blood monocytes of 5 dogs with canine ehrlichiosis at Pasteur Institute of Algeria. *E. canis* infection gained recognition as a disease of dogs. The disease gained attention during the Vietnam conflict in the late 1960s because more than 200 dogs belonging to the US military forces died as a result of an epizootic of highly fatal hemorrhagic disease known as tropical canine pancytopenia (Walker *et al*., 1970). Development of the disease was associated with heavy infestations of *Rhipicephalus*

sanguineus(the brown dog tick), which is the primary vector of *E. canis* (Groves *et al*., 1975). The age, breed, and immune competence of dogs are believed to influence the severity of infection, and some affected dogs may not have clinical signs of illness (Woddy and Hoskins, 1991). One documented case of human infection with *E. canis* has been reported in the medical literature (Perez *et al*., 1996). *E. canis* was isolated from the blood of a healthy adult in Venezuela who reported close contact with an *E canis*-infected dog (Perez *et al*., 1996). Although morulae were observed on cytologic examination of a blood smear from this individual, the infection did not result in clinical signs.

2.2.1 Morphology

Ehrlichia canis forms microcolonies within a membrane-lined intracellular vacuole (so-called morula) primarily in monocytes and macrophages of mammalian hosts (de Aguiar D.M, 2017). The pathogen replicates only in the cytoplasm of monocytic cells, and the formation of morulae is a defining characteristic (Straube, 2010). Morulae appear as stippled dark blue or purple inclusions mainly in monocytes or lymphocytes (Centre for Food Security and Public Health, 2013). Only a small percentage of the cells are infected (Centre for Food Security and Public Health, 2013). Wright, Giemsa or Diff-Quik stained peripheral blood, bone marrow smears, lymph node smears or other impression smears from fresh tissues may be used to search for morulae (Centre for Food Security and Public Health, 2013).Morulae are most likely to be found early in the acute illness and are rarely detected in chronic CE (Dumler *et al*., 1995).

2.2.2 Transmission

Ehrlichia are transmitted by ticks in the family *Ixodidae* (Centre for Food Security and Public Health, 2013). Typically, tick nymphs or larvae are infected with *E. canis* after feeding on a persistently infected dog (Harrus et al., 1988).

Rhipicephalus sanguineus, the Brown dog tick is the primary vector for *E. canis* which is transmitted transtadially but not transovarially (Groves *et al*., 1975) and all feeding stages can transmit the infection to susceptible dogs with adults being able to transmit *E. canis* for at least 155 days after detachment from an infected host (Stich *et al*., 2002). It has been confirmed that *R. sanguineus* transmits *E. canis* in dogs in Nigeria and the disease is known to be enzootic (Saror and Pimentel, 1972; Leeflang *et al*., 1976; Schillhorn Van Veen and Adeyanju, 1979; Trimnell *et al.,* 1989; Abdullahi *et al*., 1990).

Other canids may be infected with *E. canis* including wolves, foxes, coyotes, jackals and African wild dogs (Van Heerden, 1979). A natural reservoir of infection is maintained in both wild and domestic canids, including but not limited to dogs, wolves, coyotes and foxes (Claudio *et al*., 2004) The failure of canids to completely clear *E. canis* could be the important mechanism of this ongoing persistence and should be considered when selecting canine blood donors from endemic areas (Straube, 2010).

Transmission of the disease has also been reported via blood transfusion (Ettinger *et.al*., 1995) and bone marrow transfer procedures (Centre for Food Security and Public Health, 2013). Transmission via contaminated needles and mechanical transmission by biting insects are also theoretically possible (Centre for Food Security and Public Health, 2013).

It has been shown that *E. canis* can be transmitted transtadially in *Dermacentor variabilis*, with adults also transmitting the infection to dogs (Johnson *et al*., 1998). To date, there is no evidence of direct transmission of *Ehrlichia spp* from dogs to humans (Fishbien *et al*., 1987a and Fishbien *et al.*, 1987b), and dogs have been established as a reservoir for human infection (Fishbien *et al*., 1987a and Fishbien *et al.*, 1987b).

2.2.3 Host-Range and geographical distribution

Ehrlichia canis can cause illness in dogs and other canids, and these animals are thought to be the reservoir hosts. Evidence of infection with this or similar organisms have also been reported in cats and captive wild felids (Centre for Food Security and Public Health, 2013). An *E. canis*-like organism in South American felids might be a novel species (Centre for Food Security and Public Health, 2013).

The first human infection with *E. chaffeensis* was diagnosed in 1986, raising the awareness of *Ehrlichia spp* as zoonotic pathogens (Maeda *et al*., 1987). Currently, *E. canis*, *E. chaffeensis* and *E. ewingii* are all known to cause ehrlichiosis in humans. *E. canis* has been isolated in culture and detected in several human patients with overt clinical signs in Venezuela (Perez *et al*., 1996 and Perez *et al*., 2006). However, its' significance as a human pathogen is not clearly defined at this point.

Ehrlichia canis occurs worldwide, although their distribution within an area varies with the presence and density of their tick vectors (Centre for Food Security and Public Health, 2013). *E.canis* organisms are found in all continents throughout the world but are more prevalent in tropical and subtropical climates (Straube, 2010). With increasing global mobility of dogs, a

diagnosis of ehrlichial infection should not be ruled out in non-endemic areas particularly given the chronic state of the disease (Straube, 2010).

The seroprevalence indicators of *E. canis* infection show that it is very high throughout Africa, where it may be considered enzootic (Dumler et al., 2001). The prevalence of *E. canis* in dogs in African countries was reported. In Zaria, Nigeria as 6.8% (Lawal *et al*., 2001) and 11% in Jos (Joshua *et al*., 2013), Cameroon, where infection rates were 6 to 21% (Ndip *et al*., 2005), Ivory Coast where the infection rate was 27% (Socolovschi *et al*., 2012). Other African countries include: Gabon, 28% (Davoust *et al*., 2006), Senegal, 76% (Davoust *et al*., 2003), Algeria, 68% (Azzag *et al*., 2015), Chad, 28% (Brouqui *et al.,* 1991), Djouboti, 47% (Davoust *et al*., 2003), Zimbabwe 34% (Kelly *et al*.,2004), South Africa, 42% (Pretorius and Kelly, 1998), Egypt, 33% (Botros *et al*., 1995), Tunisia, 54% (M'ghirbi *et al.,* 2009).

In Asian countries, a prevalence rate of CE ranging from 0.2 to 30% (Inokuma *et al*., 1999; Rajamanickam *et al*., 1985; Stitch *et al*., 2008) was reported while in Europe, a prevalence ranging from 2 to 50% have been reported (Solano-Gallego *et al*., 2006; Cocco *et al*., 2003; Pusterla *et al*., 1998; Sainz *et al*., 1995, Salinas-Melendez *et al*., 2015). In Brazil, a prevalence rate of 9.4% (Braga *et al*., 2014) was found in cats.

Ehrlichia canis infection has been reported to be fatal in exotic breeds of dogs in Nigeria particularly the Alsatians while Nigerian indigenous breeds are said 'to be more or less' resistant (Schillhorn van Veen and Adeyanju, 1979). Leeflang *et al.,* (1976) however reported that Nigerian indigenous dogs succumb to experimental *E. canis* infection. Adeyanju and Abdullahi (1986) reported that Nigerian indigenous dogs are less severely infected than Alsatians. No sex or age predisposition has been attributed to the disease. The climatic and topographical sites of

Zaria are such that favours all year propagation of ectoparasites which harbour and transmit parasitic pathogens from one susceptible host to another (Natala *et al.*, 2009).

2.3 Clinical Signs/Presentation

Clinical signs and the severity of illness seen with ehrlichiosis depend on species of *Ehrlichia* involved and the immune response of the dog (Cohn, 2003). In general, all breeds of dogs are susceptible to *E.canis* infection, but German Shepherds seem to develop severe forms of the disease more frequently than other breeds (Nyindo *et al.*, 1980).

Canine ehrlichiosis is characterized by three stages; acute, subclinical and chronic stages. These can be difficult to distinguish in practice (Straube, 2010). Concurrent infections with other organisms may increase the severity of the disease (Centre for Food Security and Public Health, 2013).

After an incubation period of 1-3 weeks, experimentally infected dogs enter the acute phase of infection and may show depression, lethargy, anorexia, mild weight loss, fever, lymphadenomegaly and splenomegaly, although in many cases signs are mild or inapparent (Anderson *et al.*, 1991; Breitschwerdt *et al.*, 1987; Buhles *et al.*, 1974; Iqbal and Rikisha, 1994; Van Heerden J, 1982; Waner *et al.*, 1999a), Platelet-related bleeding may be observed (Harrus *et al.*, 1997a) though, unusual. Most dogs survive the acute phase of infection and recover within 1-4 weeks to enter the subclinical phase of the disease, where they show no clinical signs but remain infected with *E.canis* (Buhles *et al.*, 1974 and Van Heerden J, 1982). This subclinical phase may last for as little as 4 months in experimentally-infected dogs (Buhles *et al.*, 1974) but may persist for up to 10 years in naturally-infected dogs (Bartsch and Greene, 1996).

A significant finding is that dogs can spontaneously eliminate *E. canis* infections during the subclinical phase of the disease (Harrus *et al*., 1998; Breitschwerdt *et al*., 1998a). Similarly, it has been suggested that naturally infected dogs (68%) in Zimbabwe that were serologically positive but had no clinical, haematological or biochemical signs of infection may have self-cured (Mathewman *et al*., 1993). Spontaneous elimination of *E. canis* infections in naturally-infected dogs may therefore not be uncommon.

In some dogs, a severe, life threatening chronic phase of the disease may develop. In this phase, dogs exhibit clinical signs including weight loss and emanciation, fever, pallor, weakness, haemorrhage, and peripheral oedema, particularly of the hind limbs and scrotum (Buhles *et al*., 1974; Greene and Harvey, 1990; Harrus *et al*., 1997a; Hibler *et al*., 1986). Death usually results from extensive haemorrhage, or is due to secondary bacterial infections (Buhles *et al*., 1974; Greene and Harvey, 1990; Harrus *et al*., 1997a; Hibler *et al*., 1986).

In naturally infected dogs in which the stage of infection is not readily determined, depression, weight loss, anorexia, haemorrhagic tendencies, in particular epistaxis, pyrexia and lymphadenomegaly are the most commonly reported signs in the USA (Woody and Hoskins, 1991). Similar signs have been reported in studies on naturally-infected dogs in Africa (Mathewman *et al.*, 1993, Price and Dolan, 1980; Van Heerden, 1982). Neuromuscular, reproductive and ocular signs may also occur in naturally-infected dogs. These signs include polymyositis, paresis, signs of meningoencephalitis, cranial nerve deficits, seizures, abortions and infertility, corneal opacity, anterior uveitis, hyphema, focal chorioretinal lesions and retinal detachment (Harrus *et al*., 1997b). Pulmonary signs including coughing and exercise intolerance may also develop as a result of interstitial lung infilterates (Hibler *et al*., 1986).

Reasons proposed for the wide variations in clinical signs and the development of the severe life-threatening chronic phase of the disease in only some dogs include strain variation in *E. canis*, dose of infection, concurrent diseases and immunological status of the host (Harrus *et al*., 1997b; Hibler *et al*., 1986; Seamer and Snape, 1972). German shepherd dogs and their crosses are particularly likely to show more severe signs of disease and infections are associated with a poorer prognosis (Harrus *et al*., 1997b; Hibler *et al*., 1986; Seamer and Snape, 1972). It is very likely that *E. canis* causes immunosuppression but currently little is known about the immunobiology of this infection. A study in dogs was unable to demonstrate a marked immunosuppression (Hess *et al*., 2006).

2.4 Haematology/ Biochemistry

Thrombocytopenia appears around day 10 and peaks in the third week post-infection, with platelet counts ranging from 20,000 to 52,000/µl (normal range: 200,000– 450,000/µl). There can also be mild anemia and leucopenia (Kelly, 2000). In endemic regions, platelet counts on a blood smear are used as a screening test for CE (Bulla *et al*., 2004).

In the acute phase of the experimentally-induced disease, the most common laboratory abnormality is thrombocytopaenia (platelet counts down to 20–100 × 10^3/µl) (Anderson *et al*., 1991; Breitschwerdt *et al*., 1998b; Iqbal and Rikisha, 1994; Seamer and Snape, 1972; Waner *et al*., 1997) with an increase in platelet volume suggesting active thrombopoesis (Waner *et al*., 1997). Other abnormalities that are reported less frequently include anaemia and leukopaenia (Anderson *et al*., 1991, Buhles *et al*., 1974; Van Heerden, 1982). The bone marrow is commonly hypercellular in the acute phase of infection (Reardon and Pierce, 1981).

Laboratory abnormalities described for naturally-infected dogs in the subclinical phase of the disease include hyperglobulinaemia (90 %), thrombocytopaenia (50 %), absolute lymphocytosis (40 %) and absolute neutropaenia (30 %) (Codner and Farris-Smith, 1986). In experimentally-infected dogs, thrombocytopaenia ($13–180\times10^3/\mu l$) was observed in most cases and mean platelet volumes were increased (Waner *et al*., 1997). There were significant decreases in leukocyte and neutrophil numbers compared with pre-infection values. Similarly, although none of the dogs became anaemic, some dogs had reduced packed-cell volumes , red-cell counts and haemoglobin concentrations compared to pre-infection levels (Waner *et al*., 1997). The dogs also had increased total serum proteins (33 %), hypoalbuminaemia (22 %), hypergammaglobulinaemia (68 %), increased α 1- and α 2- (22 %) and β_1-globulins (44%) and decreased β_2-globulins (55 %) (Waner *et al*., 1997).

In experimental studies on the chronic phase of the disease, laboratory abnormalities included regenerative or nonregenerative anaemia (red cell count $2–5 \times 10^6/\mu l$), severe leukopaenia (white cell count $<3 \times 10^3/\mu l$) and thrombocytopaenia (platelet count $<30\times10^3/\mu l$) (Buhles *et al*., 1974). In the early stages, bone marrow hyperplasia occurs, but as the disease progresses, the bone marrow becomes hypoplastic (Ristic and Holland, 1993).

In naturally-infected dogs in the USA in which the stage of disease could not be determined, laboratory abnormalities included thrombocytopaenia (86 %), non-regenerative anaemia (57 %), hypoalbuminaemia (43 %), hyperglobulinaemia (39 %), hyperproteinaemia (33 %), leukopaenia (31 %), leukocytosis (20 %), pancytopaenia (17 %), and regenerative anaemia (15 %) (Woody and Hoskins, 1991). Elevated liver enzymes were found in 35 % of dogs, but prior corticosteroid usage could have been responsible for these elevations in some dogs (Sainz et al., 2015). Similar abnormalities have been reported for dogs in Africa (Mathewman *et al*., 1993; Price and Dolan,

1980; Van Heerden J, 1982). Generally, there are significantly decreased α_-1 globulins and significantly elevated _α-2, β_-2 globulins and γ_-globulins (Green and Harvey, 1990; Hibler *et al*., 1986; Woody and Hoskins, 1991). It has also been found that dogs that were pancytopaenic had significantly lower concentrations of total protein, total globulin and _γ-globulins, indicating severely compromised immune function (Harrus *et al*., 1996).

Hypoalbuminemia, hyperglobulinemia, and hypergammaglobulinemia (mostly polyclonal, rarely monoclonal) are common in *E. canis* infection. Also moderate increases in alanine aminotransferase (ALT) and alkaline phosphatase (ALP) can occur due to hepatocyte damage during the acute phase (Straube, 2010).

Dogs in the subclinical phase are clinically healthy, but variable degrees of thrombocytopenia and leukopenia may be present. Thrombocytopenia usually becomes severe in the chronic phase accompanied by marked anemia and leucopenia (Straube, 2010). Pancytopenia due to bone marrow hypoplasia is characteristic of the chronic severe form (Harrus *et al*., 1997b).

A hypocellular bone marrow with varying supression of the erythroid, myeloid, and megakaryocytic cells is seen on aspiration (Harrus *et al*., 1997b). *E. canis* can occasionally induce a protein losing nephropathy as a result of immunecomplex glomerulonephritis with consequent proteinuria and azotemia (Straube, 2010).

2.5 Pathogenesis and Pathology

Monocytes attracted to the site of tick attachment become infected with *E. canis* present in the salivary gland secretions of the tick (Greene and Harvey, 1990). Infected monocytes enter the

blood stream and lymphatics and localise in tissues throughout the body (Kelly, 2000). The persistence of the organism in these cells results in the typical histological findings of plasmacytosis and generalised perivascular lymphocyte and plasma cell accumulation (Hildebrandt *et al*., 1973). The continued presence of *E. canis* in the body results in the production of reactive IgA, IgM and IgG, and it has been suggested that these antibodies may enhance the uptake of *E. canis* into macrophages (Ristic and Holland, 1993). Dogs become susceptible to reinfection with *E. canis* only when existing infections are cleared by appropriate therapy (Anderson *et al*., 1991; Breitschwerdt *et al*., 1998a), although high antibody titres may be present.

German shepherd dogs are known to show more severe disease when infected with *E. canis* and laboratory studies have indicated that infections of these dogs cause specific and non-specific suppression of their cell-mediated immune responses (Nyindo *et al*., 1980). Anaemia in dogs with *E. canis* infections results from haemorrhage and/or bone marrow suppression. Although erythrophagocytosis is prominent in the lymph nodes, this is not a feature in other organs, and the erythrophagocytosis is thought to result from haemorrhage rather than sensitisation of red blood cells (Hildebrandt *et al*., 1973). The hypergammaglobulinaemia that is commonly found in dogs with *E. canis* infections is not due to antibodies against *E. canis,* and infections may result in non-specific antibody production (Harrus *et al*., 1997b; Ristic and Holland, 1993; Woody and Hoskins, 1991). Hypoalbuminaemia appears not to be due to renal losses, as glomerulonephritis is not common in dogs with *E. canis* infections (Codner *et al*., 1992; Hildebrandt *et al*., 1973). It may, however, result from haemorrhage, vasculitis and oedema, increased catabolism of the protein during pyrexia and/or decreased production to compensate for the oncotic effects of the hyperglobulinaemia (Harrus *et al*., 1997b).

Thrombocytopaenia is the commonest laboratory abnormality in dogs with *E. canis* infections, and there are numerous possible causes for this abnormality. In the chronic phase of infection, thrombocytopaenia is most often due to bone marrow hypoplasia (Hildebrandt *et al.,* 1973). Another possible cause of thrombocytopaenia is the production of a platelet migration inhibition factor that enhances platelet sequestration, particularly in the spleen (Adawa *et al.,* 1992). Previous experiments have shown that the spleen plays an important role in the pathogenesis of *E. canis* infections, with splenectomised dogs having less severe clinical signs and laboratory abnormalities than intact dogs (Harrus *et al*., 1998c). Inflammatory mediators from the spleen and/or other splenic substances have been proposed to play a key role in the pathogenesis of the disease.

2.6 Diagnosis

Diagnosis of *E. canis* is based on demonstrating either the morulae or parasite antigens or antibodies directed against the *E. canis* parasite (Harrus and Waner, 2011). Because thrombocytopenia is a relatively consistent finding in stages of the disease, a platelet count is an important screening test (Harrus and Waner, 2011). Confirmatory diagnosis is based on detection of typical intracellular *E. canis*-morulae on blood smear examination (Kelly, 2000). However, this method is time-consuming and not very reliable because morulae are only found in low numbers in blood smears during the acute phase of infection (Kelly, 2000). Microscopy has an estimated sensitivity of 4% (Woody and Hoskins, 1991).Detection of morulae can be improved by evaluation of numerous buffy coat smears (Mylonakis *et al*., 2003). Also, morulae of dogs with acute granulocytic ehrlichioses are indistinguishable.

Infections with *E. canis* can be diagnosed by isolation of organisms from whole blood in tissue culture (Dawson *et al*., 1991). It is possible to culture *Ehrlichia* species in specific macrophage cell lines (canine macrophage cell line [DH82] or mouse macrophage cell line [J774.A1]). However, this technique is used more in research laboratories than for diagnosis in practice (Iqbal *et al*., 1994; Dumler *et al*., 1995). While this is a sensitive method of detecting infections, the procedure is time consuming, costly, and may take as long as 2 months (Iqbal *et al*., 1994; Dumler *et al*., 1995), which reduces its clinical usefulness. Although a short-term cell culture isolation technique has been described, using monocyte cultures from the infected dog and which gives results in 4 days (Price and Dolan, 1980), the sensitivity and specificity of the test has yet to be determined.

The indirect fluorescent antibody test (IFAT) is recommended to confirm a diagnosis of ehrlichiosis (Waner *et al*., 2007). Detection of specific IgG antibodies indicates previous exposure to the ehrlichial pathogen, and during the acute disease two tests one to two weeks apart will show rising antibody titers. However, there is extensive serologic cross-reactivity between *E.canis* and *E. chaffeensis* and *E. ewingii* (Cardenas *et al.*, 2007)Thus, results obtained by IFAT need to be interpreted carefully. Low IFAT titers are of low specificity.

Enzyme-linked immunosorbent assays (ELISA) can also be used to confirm a diagnosis of ehrlichiosis and different Dot-ELISA kits for the detection of *E. canis*-IgG antibodies are commercially available. Western immunoblot is a more specific test, which can distinguish between infections with the different organisms causing ehrlichiosis, anaplasmosis, or neorickettsiosis as well as between *Ehrlichia* spp., for example *E. canis* and *E. ewingii* and hasbeen reported to be most useful for differentiation between acute and chronic *E.canis* infectionsor in cases where IFA serology is inconclusive (Hegarty *et al*., 1997). A dot-blot

enzyme-linked immunoassay(DBELIA)using purified *E. canis* antigens (Cadman *et al*., 1994)or a recombinantP30 protein of theorganism (Ohashi *et al*., 1998)that is as sensitive as IFA in the detection of antibodies against *E. canis* in experimentally- and naturally infected dogs has been described. Commercially available DBELIA kits can now be used in-house to detect antibodies reactive with *E. canis.* Also, an enzyme-linked immunosorbent assay (ELISA) (Rikisha, 1991a) has been described that may also become useful in the in-house diagnosis of *E. canis* infections. The DBELIA and ELISA would, however, be expected to have similar limitations to those described for IFAs.

Polymerase Chain Reaction (PCR) techniques are now considered to be the most reliable method to diagnose ehrlichial infection (Harrus and Waner, 2010). PCR methods are highly sensitive and enable the detection of *Ehrlichia* DNA as early as 4–10 days post-infection prior to sero-conversion (Iqbal *et al*., 1994). Numerous conventional and real-time PCRs are available based on different gene sequences. PCR can be performed on whole blood, splenic aspirates, lymph nodes, or bone marrow (Iqbal *et al*., 1994). The spleen is the organ most likely to harbor *E. canis* parasites during the subclinical phase (Harrus *et al*., 1997a) and is considered to reveal higher sensitivity than testing of bone marrow or blood samples (Harrus *et al*., 1998a; Harrus *et al*., 2004). Although PCRs are extremely sensitive and specific in identifying infections with the different *Ehrlichia* species in dogs, their use is currently restricted to research laboratories.

2.7 Treatment

Treatment of *E. canis* infections is considered to be successful when dogs recover clinically, the haematology and biochemistry values return to normal and the organism can no longer be shown to be present in the body (Kelly, 2000).

Tetracyclines have proven effective against *E. canis* infections in dogs (Kelly, 2000). Based on the fact that tetracyclines are known to be generally effective against all rickettsias and that they are effective against *E.canis* in most patients, tetracyclines remain the drug of choice in the treatment of canine ehrlichioses (Kelly, 2000). Tetracyclines act by inhibiting protein synthesis at the 30S ribosomal subunits of bacteria (Hash, 1972). Of the tetracyclines, doxycycline is probably the most suitable for use in dogs, as it has higher lipid solubility than the other tetracyclines and it is thus better absorbed from the gastrointestinal tract and penetrates tissues better (Shaw and Rubin, 1986). Treatment with doxycycline is recommended at 10 mg/kg orally daily for at least 2–6 weeks (Kelly, 2000). Oxytetracycline and tetracycline HCl are recommended at 22 mg/kg given every eight hours daily for 4 weeks (Harrus *et al*., 1998b; Harrus *et al*., 2004).

Doxycycline, has a long half-life (12 hours), and can be given at lower doses and less frequently than other tetracyclines, which would be expected to improve owner compliance in administering the drug (Kelly, 2000). Also, doxycycline is less likely to induce vomiting in dogs, which has been reported to be a common side effect of tetracycline HCl therapy (Price and Dolan, 1980). Doxycycline, unlike other tetracyclines, does not seem to cause enamel discoloration in puppies (Sainz *et al*., 2015). Moreover, one of the most common side effects of tetracyclines, vomiting, can be eliminated by splitting the antibiotic dose into two half-doses

every 12 h (q12h) or by administering the antibiotic after feeding (Sainz *et al*., 2015). When the dog has liver problems, the use of doxycycline should be reconsidered. In any case, liver function tests should be performed before and during treatment; when liver parameters increase, the treatment regime should be stopped (Wen *et al*., 1997; Harrus *et al*., 1998b; Neer *et al*., 2006; Little, 2010)

Terramycin LA (Long acting) has been found to provide a sustained antibacterial activity over a period of 3.5 days from a single intramuscular injection in cattle (Breeze and Gay, 1981; Baldwin, 1978). The giving of two injections at a four-day interval means that at least 6-10 days of high blood plasma concentrations of oxytetracycline must have been achieved. This prolonged concentration of oxytetracycline has been found to be effective against *E. canis* (Ogunkoya *et al*., 1985). With this approach, repeated calls of the clients for 6-14 successive days at the clinic have been reduced to two and the chances of fluctuations of therapeutic blood levels that are common when other Terramycin groups of drugs are used have also been considerably reduced. The above resulted in fewer acute cases of canine ehrlichiosis becoming chronic (Ogunkoya *et al*., 1985).

Other drugs have been historically used for treating *E. canis* infection. Chloramphenicol has been used in dogs under 1 year of age (Sainz *et al*., 2015), but its use is not recommended when doxycycline is available (Sainz *et al*., 2015). Imidocarb dipropionate has also been described as a potential treatment for ehrlichiosis in dogs (Price and Dolan, 1980; Mattewman *et al*., 1994; Sainz *et al*., 2000). Recent studies performed *in vitro* and in experimentally infected dogs showed that imidocarb dipropionate was not effective against *E. canis* (Kelly *et al*., 1998; Eddlestone *et al*., 2006). It should only be used in cases of co-infection with *Babesia canis* or

Hepatozoon canis (Sainz *et al*., 2015). Other antibiotics, such as rifampicin or levofloxacin, have been effective in *in vitro* studies (Schaefer *et al*., 2008). However, previous studies have shown that, in experimental infections, rifampicin contributed to improving laboratory findings, but it was not effective in eliminating the infection (Theodorou *et al*., 2013).

2.7.1 Supportive therapy

Supportive therapy such as blood or fluid transfusions and anabolic steroids may be required in severe cases (Sainz *et al*., 2015). The prognosis becomes poor once dogs enter the chronic phase of disease (Mylonakis *et al*., 2004). In animals with life-threatening, severe anaemia, blood transfusions should be administered. Fresh whole blood or platelet-rich plasma is indicated in dogs with life-threatening haemorrhage (Kelly, 2000). Multiple transfusions may be required before adequate bone-marrow responses occur, and it is important in such cases that crossmatching be performed to prevent transfusion reactions (Kelly, 2000). Concurrent use of long acting tetracycline and piroxicam in treatment of selected cases of canine ehrlichioses resulted in the abolition of pain and swelling at the tetracycline-injection sites and fever (Adawa *et al*., 1992). The study also showed rapid restoration of appetite observed in the treated dogs probably due to abolition of fever by piroxicam and the rapid elimination of *E. canis* by the tetracycline (Adawa *et al*., 1992).

Good supportive care is also indicated in dogs being treated for *E. canis* infections. This includes placing the dog on a high plane of nutrition, avoidance of environmental stress factors and treatment of concurrent diseases (Kelly, 2000).

2.7.2 Treatment failures

After initiation of treatment, a rapid improvement in clinical signs is usually seen, but several weeks of therapy are usually required to ensure a full recovery (Sainz *et al*., 2015). Persistent infections with *E. canis* often remain as complete bacterial clearance is not guaranteed but has been reported in some cases following antibiotic therapy (Harrus *et al*., 2004; Iqbal and Rikihisa, 1994; Harrus *et al*., 1998b; Breitschwerdt *et al*., 1998b; Schaefer *et al*., 2007). It has been suggested that the phase of Canine Ehrlichiosis could affect the efficacy of doxycycline treatment in clearing *E. canis* infections (Schaefer *et al*., 2007). Experimentally infected dogs treated with doxycycline for 14 days were still infectious to ticks and thus reservoirs of *E. canis* infection (Schaefer *et al*., 2007). There are numerous possible reasons for these treatment failures and incomplete responses including: lack of owner compliance in administering the drug at the correct dosage for the correct duration of therapy and not around times of feeding (Price and Dolan, 1980); dogs vomiting the tetracycline (Price and Dolan, 1980); continual reinfections of the dogs with *E. canis* in endemic areas (Breitschwerdt *et al*., 1998a); concurrent diseases maybe present that mimic or exacerbate the signs of *E. canis* infections (Breitschwerdt *et al*., 1998b); dogs being in the chronic phase of *E.canis* infections. Dogs with severely hypoplastic bone marrow have a grave prognosis, as the non-regenerative anaemias, thrombocytopaenias and/or leukopaenias generally take a long time (2–6 months) to resolve, and dogs often succumb to infections or fatal haemorrhage before recovery (Buhles *et al*., 1974; Greene and Harvey, 1990; Hibler *et al*., 1986); persistence of high antibody titres following the elimination of *E. canis* due to aberrant immune responses (Bartsch and Greene, 1996); inefficacy of tetracycline therapy owing to the persistence of *E. canis* in organs where tetracycline penetration is poor (Shaw and Rubin, 1986); concurrent long-term use of immunosuppressive drugs (Perille and Matus, 1991);

persistence of ehrlichial DNA unassociated with viable organisms (Breitschwerdt *et al*., 1998a); and the presence of concurrent infections with other ehrlichia (Breitschwerdt *et al*., 1998b; Kordick et al.,1999).

2.8 Prevention

There are no vaccines currently available to protect dogs from *Ehrlichia* spp. infections, and further research is needed to define the virulence factors and immunoprotective antigens required to develop one (McBride *et al*., 2010).

The prevention of ehrlichiosis infection in dogs must be focused on tick control. Nevertheless, to prevent transmission, actions must focus mainly on:

Preventing dogs from becoming infested in the field, which is the source of peridomestic parasitism. This infestation can introduce ticks to indoor habitats (kennels, etc.), which will result in a large population, due to their high reproductive capacity (Sainz *et al*., 2015).

Preventing dogs from becoming infested with ticks, even when they live in a peridomestic environment with abundant ticks. This objective is more difficult to achieve than the former, due to the high parasitism pressure that can arise from those populations of ticks (Dantas-Torres, 2008).

Apart from appropriate epidemiological measures, the best prevention against dog tick infestation is based on the use of ectoparasiticides. In general, these are active molecules that act against ticks, and they must also provide some degree of appropriate control within a short period of time (Pereira *et al*., 2009). Rapid action could contribute to the elimination of attached

ticks before they are able to transmit pathogens. To optimize efficacy, preventive chemicals must be applied at the recommended time intervals, based on the length of efficacy claimed by the Manufacturer. Although knowledge may be lacking for some diseases, most tick-borne pathogens require 4 to 48 hours to complete their development in the salivary gland and enter the bloodstream (Nicholson *et al*., 2010). However, a report showed that transmission of *E. canis* can occur within shorter periods of time e.g., 3 hours (Fourie *et al*., 2013).

Prevention of transfusion-associated transmission can be reduced by using seronegative screened blood donors, although new donors with a negative screen cannot be presumed free of infection for several weeks because they may be incubating infection. Prophylactic administration of tetracycline at a lower dosage (6.6 mg/kg/day, PO) is effective in preventing *E. canis* infection in kennels where disease is endemic (Merck Veterinary Manual, 2014). Treatment must be extended for many months through at least one tick season if the endemic cycle is to be successfully eliminated, and tick control should be implemented as well.

To appropriately control tick infestations in dogs requires knowledge of tick seasonality (Sainz *etal*., 2015). This seasonality is usually strict under natural conditions, and each stage of the life cycle of the tick follows the previous stage with regularity. However, these patterns can be lost in areas with large infestations in a peridomestic environment (Sainz *et al*., 2015). Under those conditions, all stages can be active simultaneously, due to the presence of several infestations in the kennel (Sainz *et al.*, 2015).

Various antiparasitic treatments are registered in Europe that have activity against ticks and can be prescribed for dogs (WHO, 2006). The specific product can be selected according to the preferences of use (collar, pour-on, or spot on) and the therapeutic needs for each case. Some

compounds, such as the pyrethroids or some preparations of diazinon, are registered as repellents (WHO, 2006).

Treatments with ectoparasiticides that repel and kill ticks reduce the risk of disease transmission (Sainz *et al*., 2015). Spot-on products are applied topically to the dog's skin. Recent studies have evaluated the efficacy of a spot-on formulation containing imidacloprid 10% and permethrin 50% (Advantix®) to prevent tick exposure and thus *E. canis* infection in dogs. Preventive efficacies of 95–100% were demonstrated in treated dogs living under natural conditions in endemic areas (Otranto *et al*., 2010; Otranto *et al*., 2008).

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 Study Area

The study was carried out in 5 selected wards in Sabon Gari Local Government Area, Zaria. Twenty dogs were sampled from each ward. Consent of the dog owners was sought before sampling. The selected wards were Muchia, Dogarawa, Bomo, Jushi and Basawa wards, all in Sabon Gari Local Government Area (Figure 1).

Wards were selected based on convenience.

Zaria metropolis is located in Kaduna State, within latitudes 11° 7’ to 11° 12’ N and longitude 07° 41’ E. It is a medium sized city with an estimated population of 408,198 (NPC, 2006). It is divided administratively into Zaria and Sabon Gari Local Government Areas (Ministry of Economic Development, 1996). It has an estimated land area of about 300 square kilometers (ABU, 2000).

Sabon Gari Local Government Area in Kaduna state has an area of 263km^2, density of 1,287.11inh/km^2 and a population of 291,358 at the 2006 census. It posseses a tropical continental climate with a pronounced dry season lasting seven months (October to May). Rainy season starts from May to September with a long term annual rainfall of 1040 mm in about 90 rain days (ABU, 2013).

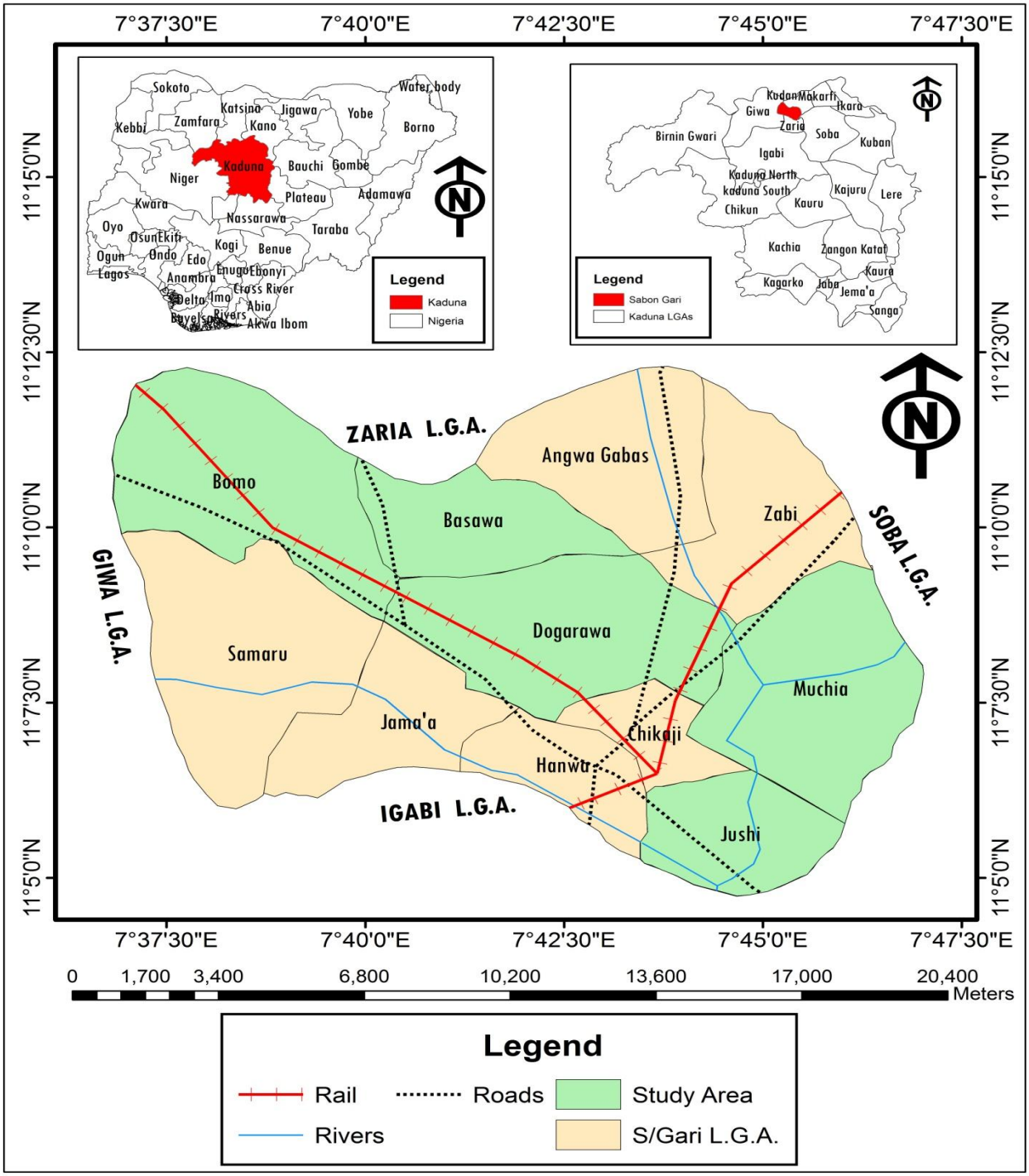

Figure 1: Map of Sabon Gari L.G.A. Kaduna State Showing the Study Area.

Source: G.I.S. Laboratory, Geography Department A.B.U. Zaria (2017).

3.2 Study Animals

The study animals were dogs sampled with consent of the owners. Seventy three percent of the dogs were free- roaming used for hunting while 27% were housed, as pets or used as security dogs.

3.3 Sample Size

The samples were obtained using the formula outline of Thrushfield (1997) at 95% confidence level as follows:

$N = Z^2 pq/d^2$

Where N = sample size

Z^2 = appropriate value for the standard normal deviate=1.96

p = anticipated prevalence

q = 1-p

d = level of significance (0.05)

Using a prevalence rate of 6.8% determined by Lawal *et al*., (2008) in previous work in Zaria, the sample size became:

$$N = \frac{(1.96)^2 x\ 0.068 \times 0.932}{(0.05)^2}$$

$$= \frac{3.8416 \times 0.063376}{0.0025}$$

$$= \frac{0.24346524}{0.0025}$$

= 97.38

A total of 100 samples were collected.

3.4 Physical Examination of Animals

Routine physical examinations, history and clinical signs preceded taking of blood samples. Information on age, breed and sex were also obtained from examination of the animals.

3.5 Sample Collection

A total of a hundred dogs were sampled. Information on age, breed and sex were also recorded.

Ten millimeters of blood was collected aseptically via cephalic venipuncture. Five millimeters was transferred into heparinized tubes (Micropoint Diagnostics, Lagos, Nigeria) for *Ehrlichia* culture, 2mls was transferred into EDTA (Biorapid Co., Nigeria) bottles and sent to Clinical Pathology and Protozoology laboratories, Veterinary Teaching Hospital, Ahmadu Bello University, Zaria for haematological and parasitological analyses respectively. The 3mls left was dispensed into plain sample bottles and left to clot. Blood samples were centrifuged at 3,000 revolutions per minute (rpm) for 3 minutes. Serum was harvested and preserved at -20^{o}C. Blood

samples that could not be analyzed immediately were stored at 4°C in a refridgerator. Sera were used for serum biochemistry analysis and for the rapid *E.canis* antibody test.

3.6 Sample Processing Techniques

The thin blood and buffy coat smears were examined for the presence of *E. canis* morulae in monocytes using the methods as described below.

3.6.1 Thin blood smear

The procedure by Anne and Gary, (2006) was carried out as follows:

A drop of blood was placed on one end of a dust and grease free-glass slide and, using a spreader and maintaining an angle of 45º, it was spread into a smooth, thin film. The blood film was air-dried, labeled and fixed in absolute methanol for 5 minutes and air-dried. It was then stained in Giemsa solution for 25 minutes and washed in running tap water before rinsing in buffered saline. The stained slide was air-dried and examined at x100 magnification using oil immersion.

3.6.2 Buffy coat smear

The procedure described by Rathore and Sengar (2005) was observed as follows:

Fresh blood from the samples was gently mixed and drawn into a microcapillary tubes by capillary action. The tube was filled to approximately ¾ of its' length. The empty end of the

filled tube was sealed with Bunsen flame, placed in a microhaematocrit centrifuge with the sealed end facing outward, and then centrifuged for 3 minutes at 3,000 rpm. A diamond marker or pencil was used to cut off the topmost plasma layer which was removed and discarded. The interface layer was then removed and a drop was smeared onto a clean, grease free glass slide and processed just as in the procedure for making thin blood smear as described in section 3.6.1 above.

3.6.3 *Ehrlichia* culture

The procedure, which is a short term cell culture isolation technique was conducted based on the modified protocol of Price and Sayer (1983) as follows:

Five milliliters of blood was drawn from the animal into sterile heparin coated tubes via cephalic venipuncture. The tubes were centrifuged for 3mins each at 3,000 rpm. Thereafter, the plasma and leukocytes were aseptically collected into Leighton (Bellco Glass®, Inc.; Part #: 1904-19105, Vineland, New Jersey) tubes (Plate I), allowing 1ml per tube and 2 tubes per each sample. The tubes were incubated at 37°C for 48hr under atmospheric conditions. One mililitre of Dulbeco's modified Eagle's medium (Sigma-Aldrich®, D6429, Lot RN BD3533; St. Loius, United States) containing 20% homologous serum without antibiotics was added to each tube. The tubes were subsequently fed with the same medium between 48 and 72 hrs. At the end of two and four days post-incubation the cover slips from the Leighton's tubes were removed, stained with Giemsa stain (as is normally done) for thin blood and buffy coat smears as described above.

3.6.4 Rapid *E.canis a*ntibody test

Sera obtained from blood from dogs in the study were used. The Sera were retrieved from the Deep Freezer and allowed to thaw at room temperature on the work bench.

The procedure was conducted according to the manufacturer's guidelines, briefly:

The test kit was removed from the foil and placed on a flat dry horizontal surface. A dropper was used as a pipette to collect the sample from the serum. Ten µl of the sample was added into a sample hole marked "S" and was observed until the sample was completely absorbed into the sample hole. Then 3 drops of the diluent from the dropper bottle containing the assay diluent was added and the results interpreted after 10-20 minutes.

After about 10-20minutes, a purple color band appeared in the left section of result window to show that the test was working properly. This band was the control band. The right section of the result window indicated the test result. The presence of only one band ("C") within the result window indicated a negative result, the presence of 2 color bands ("T" and "C") within the result window, no matter which appeared first indicated a positive result (See Plate II). Where the purple color band ("C") was not visible within the result window after the test, the results was considered invalid. Also where the color band appeared on "T" of the result window, it was invalid.

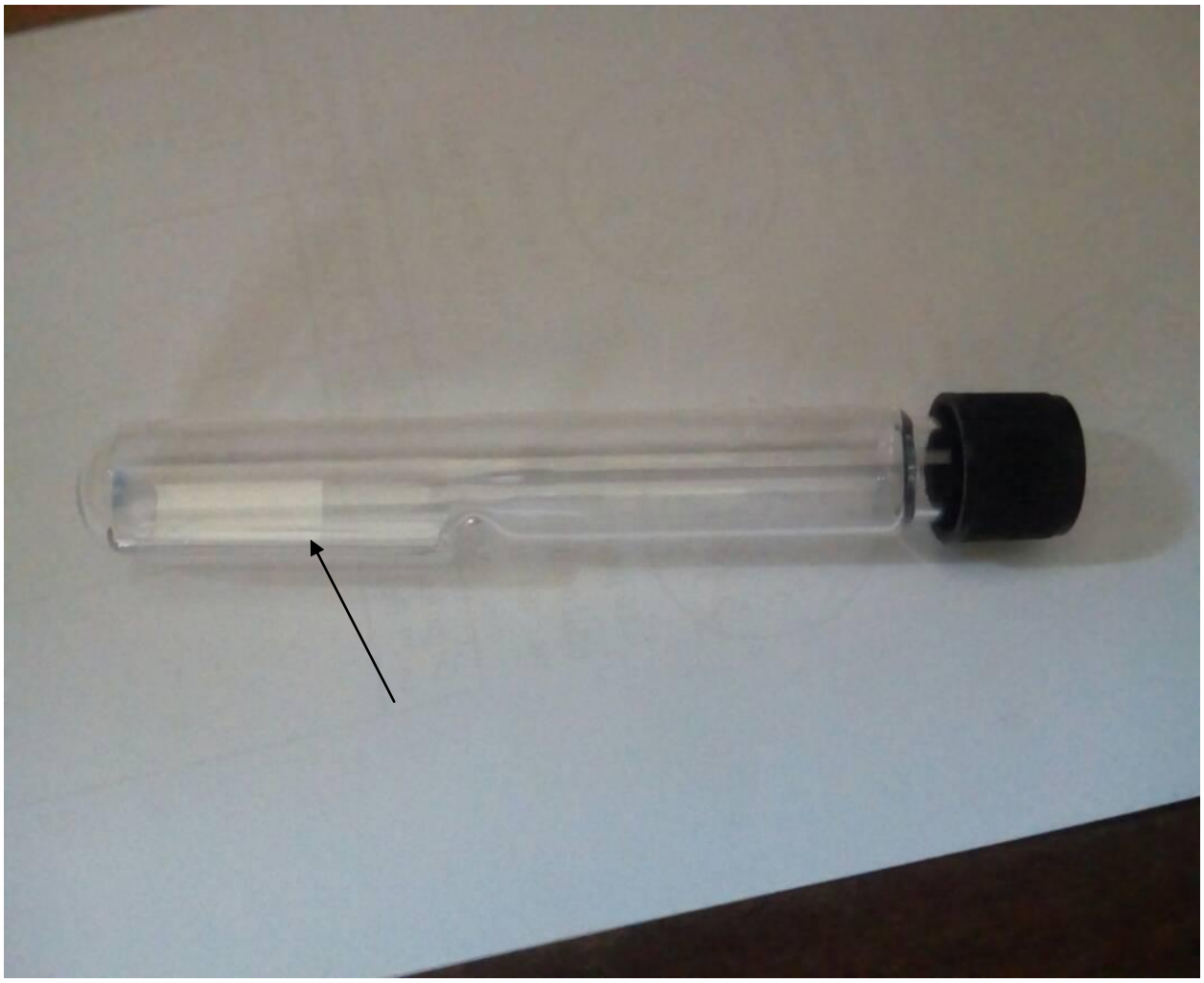

Plate I: Leighton's culture tube with its coverslip (indicated by black arrow)

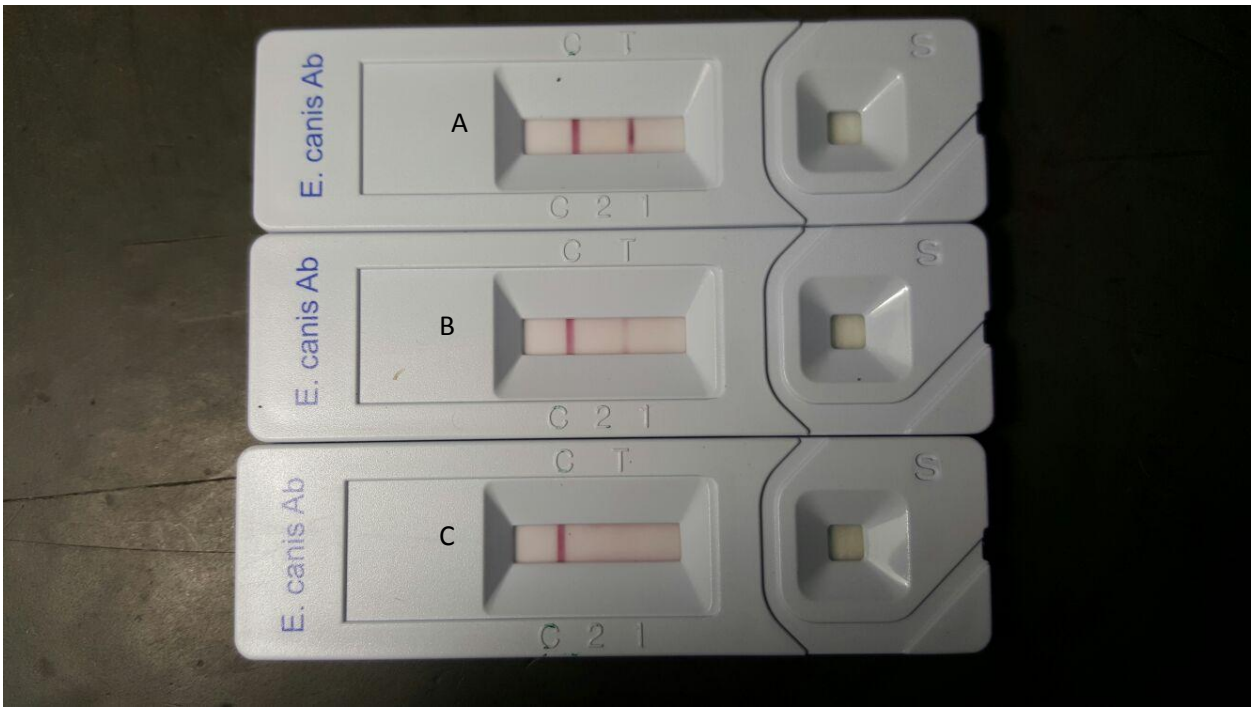

Plate II: Varying samples of rapid *E.canis* antibody test. A, shows a strong positive result, B indicates a weak positive result while C indicates a negative result.

3.6.5 Packed cell volume (PCV)

Packed Cell Volume (%) was determined using the standard microcapillary method (Coles, 1986) as described for thin blood and buffy coat smears in sections 3.6.1 and 3.6.2. The spun capillary tube was then placed on a microhaematocrit reader and the PCV read in percentage.

3.6.6 Haemoglobin concentration (Hb)

Packed Cell Volume was determined as described in section 3.6.5. Haemoglobin (g/dL) was estimated by calculation at approximately 1/3 of the PCV (Coles, 1986).

3.6.7 Total plasma protein (TP)

Total plasma protein (g/dL) was determined using refractometer method (Kerr, 1989) as follows: After determing PCV as in 3.4.5 above, the microcapillary tube containing the plasma was broken just above the buffy coat- plasma interface. The plasma was allowed to flow onto the refractometer prism. The refractometer was then held to a bright light and the reading taken at the dividing line between the bright and dark fields. The protein value given in grams per deciliter was read directly from a scale inside the refractometer.

3.6.8 Total leukocytes count

Total White Blood Cell count (10^9/L) was determined using the haemocytometer method (Coles, 1986) as follows: Whole Blood sample was drawn using a dilution pipette to the 0.5 mark. The diluent glacial acetic acid was then drawn to the 11 mark of the pipette to provide a dilution ration of 1:20. Rubber pipette closure was applied and the pipette was placed on a pipette shaker for 3 minutes to mix the blood and the diluent. The mixture was then allowed to flow into the haemocytometer to charge it. The number of white blood cells (WBC) in the 4- cornered ruled squares of the haemocytometer were counted using low power objective and the total WBC count was calculated using the formula: Total leukocytes in 4 square mm/4 x 10 = Leukocytes/µL.

3.6.9 Total platelet count

Platelet counts (10^9/L) were estimated from a blood smear examination following the procedure of Ritu *et al*., (2015) as follows: Using a well prepared thin blood smear, platelets were estimated by counting the average number of platelets seen per x100 oil immersion field in monolayer. In general, 10 oil immersion fields were counted and the results averaged (this accounts for uneven dispersal of platelets in the smear). Then the following formula was applied:

Estimated platelet count/µL = average count in 10 fields x 15000

This value was then compared to the reference range for dogs.

Reference range= 211-621 x10^9/L (Merck Veterinary Manual, 2014)

3.6.10 Biochemical parameters

Liver and kidney function tests were carried out using a semi-auto biochemistry analyzer (Rayto® RT-9200). The tests were assayed using diagnostic kits manufactured by Agappe® for serum creatinine, urea, alanine aminotransferase (ALT), alkaline phosphatase (ALP), total protein (TP), albumin and globulin.

3.6.10.1 *Alanine aminotransferase (ALT)*

ALT was determined using the methods described by Najih (1998).

Four test tubes were arranged on a test tube rack labeled: Test (T), Test blank (TB), Standard (S) and Standard Blank (SB) respectively. 0.5ml buffered substrate (alanine α- ketoglutarate) was added to all the tubes and incubated for 5 minutes. Afterward, 0.1ml of serum was added to the test (T), and then 0.1ml of pyruvate was added to the Standard (S). The solutions were mixed and incubated at 37ºC for 30 minutes, then 0.5ml dinitrophenylhydrazine (DNPH) was added to all the four tubes while 0.1ml of serum was added to test blank (TB), the solutions were mixed and allowed to stand at room temperature for 20 minutes, then 5ml of 0.4 NaOH and solutions were allowed to stand for further 5 minutes. Absorbance was read at 510nm. Value of ALT were read as

$$\text{Serum ALT activity} = \frac{T - TB}{S - SB} \times \text{Concentration of Standard} \times \frac{1000}{\text{Incubation time} \times \text{volume of sample used}}$$

(µmol/min/litre)

3.6.10.2 *Serum alkaline phosphatase (ALP)*

ALPwas determined as described by Najih (1998). 1ml of alkaline buffer was placed into a test

tube. 1ml of phenol phosphate substrate was added to the tube. The test tube was placed in a water bath (37ºC) for 5 minutes and thereafter; 0.1ml of serum was added into the tube in the incubator. The solution was mixed and incubated at 37ºC for 30 minutes. After the tube was removed from the incubator, 0.8ml of NaOH, 1.2ml of $NaHCO_3$, 1.0ml of 4- amino antipyrine and 1ml of potassium ferric cyanide were adde to it while 1.2ml of buffer was added at 1.0ml of phenol and treated as test after incubation. Absorbance was read at 510nm. Serum alkaline phosphatase activity was read as:

$$\text{Serum ALP activity (µmol/min/litre)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times 71$$

3.6.10.3 *Serum total protein (TP)*

TP was measured by the Biuret method (Coles, 1986). Serum sample (0.1ml) was mixed with 5ml Biuret reagent (as blank). 0.1ml of BSA (0.6%) was mixed with 5ml of Biuret reagent (as standard). All the above mixtures were incubated at 37ºC before the absorbances (optical densities) were read at 540nm. The results were calculated using the formula: T/5 x concentration of standard, where T= absorbance of mixture.

3.6.10.4 *Serum albumin*

Albuminwas measured colorimetrically using bromocresol green dye as described by Alberto (1996).

Three test tubes were arranged on a test tube rack. Four mls of Bromocresol green (BCG) was added to all the test tubes. 0.02 ml of distilled water was added to one of the test tubes as blank and 0.02ml of serum was added to both Test and Standard tubes. The mixtures were mixed well and absorbance was read at 628nm zeroing the instrument with the blank. Values of albumin were calculated as:

$$\text{Albumin (g/L)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times \text{concentration of Standard}$$

3.6.10.5 *Serum globulins*

Globulins were determined by subtracting the values obtained for albumins from the values obtained from total protein (Bush, 1991).

3.6.10.6 *Serum urea*

Urea was measured by the GLDH method (Kassirer, 1971). The procedure measures the concentration of urea using the urease enzyme, which converts urea to ammonia and carbon dioxide.

Serum samples were diluted in ratio 1:4 using normal saline (dilution factor=5). Five µL of the diluted serum (in duplicate) was added to the microplate wells; then 150µL of urease mix solution. Samples were mixed gently by tapping and incubated for 15 minutes at room temperature. 150µL of alkaline hypochlorite was added to each well and incubated at room temperature. The absorbance of each sample was measured in duplicate.

3.6.10.7 *Serum creatinine*

Creatininewas estimated by Jaffes method (Larsen, 1972) using the diagnostic kit.

The rate of absorbance was measured at 492nm. The samples were mixed well and kept to rest at room temperature for 20 minutes. The absorbance of the Standard and Test samples were measured against the blank. Results were calculated as follows:

Creatinine (mg/dL) = Absorbance of test/ Absorbance of sample x 200

3.7 Data Analysis

The data generated were analyzed using Chi-square tests (Steel and Torrie, 1980) and Fisher's Exact Test to determine the strength of association between the prevalence of *E. canis* infection and the various tests; as well as age, sex and breed. Fisher's Exact Test and Chi-square were also used to determine the strength of association between the diagnostic tests and the selected wards. Values of $p<0.05$ were considered significant. Kappa's test (Altman, 1991) was used to determine the level of agreement between the various tests. Values less than 0.00 were considered poor, slight from 0.00- 0.20; 0.21- 0.60 were considered as moderate, 0.61- 0.80, as good and greater than 1.0 were considered as almost perfect. The student's t-test was used to determine if the mean values of the positive and negative groups were significantly different at $p\leq0.05$. These were analyzed using the Statistical Package for Social Sciences (SPSS) for Windows Version 20.0 (IBM Corporation, Armonk, New York, United States).

Results are presented using tables and charts.

CHAPTER FOUR

4.0 RESULTS

4.1 Physical Examination of Dogs

Eighty three percent of the dogs sampled were apparently healthy. However, on physical examination 15% of the dogs had fever (39.5- 40.8 ºC), presence of ticks in 15%, rough hair coats in 10%, emanciation in 10%, diarrhoea in 1%, pale mucus membranes in 3%, worms in faeces in 1%, blood tinged urine and scrotal edema in 1%. Two percent of the dogs were reported to also have depressed appetite.

4.2 Thin Blood Smear

Examination of Giemsa-stained thin blood smears from the 100 dogs sampled revealed 0% positivity as indicated by absence of morulae in uninfected monocytes (Plate III).

4.3 Buffy Coat Smear

Examination of Giemsa-stained buffy coat smears from the 100 sampled dogs revealed 2% positivity for the morulae of *E. canis.* The morulae were compact showing intracytoplasmic organisms in the mononuclear cells. The organisms were stained purple/navy blue in colour (Plate IV).

Table 1 shows the prevalence of *E. canis* infection using the various diagnostic tests. There was no significant association seen between the various diagnostic tests and prevalence of the

disease. Table 2 shows the prevalence of the selected wards under study, using the different diagnostic tests. There was significant association (p= 0.031) found between *Ehrlichia* culture test and Dogarawa ward at a prevalence rate of 40%. The results of the investigation were also analyzed to note breed, age and sex predispositions (Table 3). There was no significant association found between age (Table 4) and thin blood/buffy coat smears (0.000 and 0.666 respectively) in relation to *E. canis* infection. In relation to breed (Table 5) and sex (Table 3), thin blood smears (0.000 and 0.989 respectively) and buffy coat smears (0.000 and 0.767) showed no significant association.

Table 6 shows the outcome of 2 of the diagnostic methods in comparism with buffy coat smear.There was a slight level of agreement between the applied diagnostic methods (buffy coat smear vs *Ehrlichia* culture; 0.151; p< 0.04 and buffy coat smear vs serology; 0.008; p< 0.518). However, there was a significant association between buffy coat smear and *Ehrlichia* culture test (p= 0.04). Table 7 also indicates a slight level of agreement between serology and *Ehrlichia* culture test (0.069; p< 0.110).

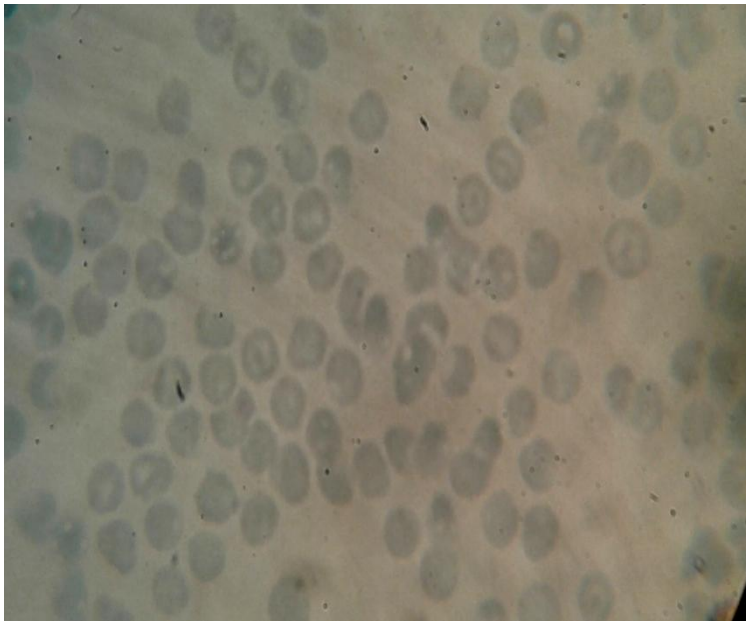

Plate III: Giesma stained thin blood smear showing absence of *Ehrlichia canis* infection.

Magnification = x5000 (100 objective x 10 eyepiece x 5x optical zoom)

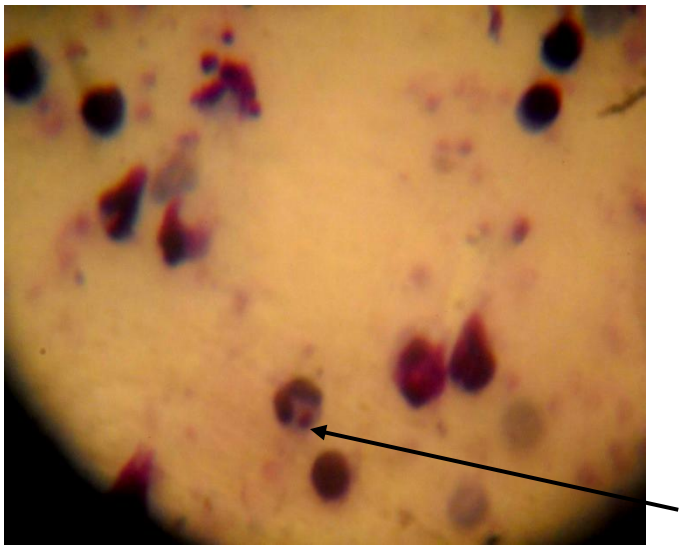

Plate IV: Intracytoplasmic morula of *E. canis* in an infected monocyte (black arrow) using buffy coat smear technique.

Magnification = x5000 (100 objective x 10 eyepiece x 5x optical zoom)

Table 4.1: Prevalence of *Ehrlichia canis* infection using various diagnostic tests

Tests	Total no. sampled	No. positive	Prevalence (%)
Thin blood smear (TBS)	100	0	0.0
Buffy coat smear (BCS)	100	2	2.0
***Ehrlichia* culture (EC)**	100	20	20.0
Rapid *E.canis* Ab test	100	83	83.0

Fisher's Exact Test= 234.344; df = 3; p = 0.000; Ab = Antibody

Table 4.2: Prevalence of *Ehrlichia canis* infection in the selected wards using the different diagnostic methods

Wards	Total no. sampled	BCS Positive (%)	EC Positive (%)	Rapid *E. canis* Ab test Pos (%)
Dogarawa	20	0 (0.0)	8 (40.0)	17 (85.0)
Jushi	20	1 (5.0)	3 (15.0)	17 (85.0)
Bomo	20	1 (5.0)	6 (30.0)	16 (80.0)
Muchia	20	0 (0.0)	2 (10.0)	15 (75.0)
Basawa	20	0 (0.0)	1 (5.0)	18 (90.0)
Total	100	2 (2.0)	20 (20.0)	83 (83.0)
x^2		3.061*	10.625*	1.843
p value		0.999	0.039	0.765

x^2= chi square test; *= Fisher's exact test; BCS = Buffy coat smear; EC = *Ehrlichia* culture

Table 4.3: Sex related prevalence of *Ehrlichia canis* infection across tests

Sex	Total no. sampled	TBS positive (%)	BCS positive (%)	EC positive (%)	Rapid *E.canis* Ab test positive (%)
Male	62	0 (0.0)	1 (1.6)	12 (19.4)	52 (83.9)
Female	38	0 (0.0)	1 (2.6)	8 (21.1)	31 (81.6)
Total	100	0 (0.0)	2 (2.0)	20 (20.0)	83 (83.0)
x^2			0.125*	0.042	0.088
p-value			0.738	0.837	0.767

x^2= Chi-square test; *Fishers exact test; BCS = Buffy coat smear; TBS = Thin blood smear; EC = *Ehrlichia* culture

Table 4.4: Age related prevalence of *Ehrlichia canis* infection across tests

Age (Years)	Total no. sampled	TBS positive (%)	BCS positive (%)	EC positive (%)	Rapid *E. canis* Ab test positive (%)
Less than 1	11	0 (0.0)	0 (0.0)	2 (18.2)	4 (36.4)
1 – 3	58	0 (0.0)	1 (1.7)	10 (17.2)	49 (84.5)
4 – 6	21	0 (0.0)	1 (4.8)	7 (33.3)	20 (95.2)
7 – 9	8	0 (0.0)	0 (0.0)	1 (12.5)	8 (100.0)
10 and above	2	0 (0.0)	0 (0.0)	0 (0.0)	2 (100.0)
Total	100	0 (0.0)	2 (2.0)	20 (20.0)	83 (83.0)
x^2			3.511*	2.913*	15.629*
p-value			0.6660	0.5440	0.0010

x^2= Chi-square test; *Fisher's exact test; BCS = Buffy coat smear; TBS = Thin blood smear; EC = *Ehrlichia* culture

Table 4.5: Breed related prevalence of *Ehrlichia canis* infection across tests

Breed	Total no. sampled	TBS positive (%)	BCS positive (%)	EC positive (%)	Rapid *E.canis* Ab test positive (%)
Local	77	0 (0.0)	2 (2.6)	16 (20.8)	65 (84.6)
Cross	12	0 (0.0)	0 (0.0)	4 (33.3)	12 (100.0)
Exotic	11	0 (0.0)	0 (0.0)	0 (0.0)	6 (54.6)
Total	100	0 (0.0)	2 (2.0)	20 (20.0)	83 (83.0)
x^2			5.633*	4.113*	8.879
p-value			0.989	0.128	0.019

x^2= Chi-square test; *Fisher's exact test; BCS = Buffy coat smear; TBS = Thin blood smear; EC = *Ehrlichia* culture

Table 4.6: Outcome of results of *Ehrlichia* culture and Serology compared to Buffy Coat Smear

		Ehrlichia culture		Serology	
BCS	Total	Pos	Neg	Pos	Neg
Pos	2 (100.0)	2 (100.0)	0(0.0)	2 (100.0)	0(0.0)
Neg	98 (100.0)	18 (18.4)	80 (81.6)	81 (82.7)	17 (17.3)
Total	100 (100.0)	20 (20.0)	80 (80.6)	83 (83.0)	17 (17.0)
k value		0.151		0.008	
p-value		0.004		0.518	

k = kappa's test; BCS = Buffy coat smear

Table 4.7: Outcome of results of Serology in comparism with *Ehrlichia* culture

	Ehrlichia Culture			
		Positive	Negative	Total
Serology	Positive	19 (22.9)	64 (77.1)	83 (100.0)
	Negative	1 (5.9)	16 (94.1)	17 (100.0)
	Total	20 (20.0)	80 (80.0)	100 (100.0)
k value		0.069		
p-value		0.110		

k= kappa's test

4.4 Serological Analysis

Sera of 83 dogs (83%) revealed positive results as demonstrated by development of a second purple line/band in the "test" window (Plate I). The dogs that revealed presence of organisms in their buffy coat smears were positive. In addition, eighty-one dogs that did not reveal organisms in their buffy coat smears were positive of *E. canis* infection using the rapid Antibody test.

Analysis of results of the serological test with respect to sex of the dogs (Table 2) revealed a higher occurrence in male dogs (52%) than females (31%) with prevalence of breed (Table 5) higher in Local dogs (65%) than Cross breeds (12%). The Exotic breed had the least prevalence of 6%. Analysis of the serological test with respect to age of the dogs (Table 4) revealed the highest prevalence in dogs of 1-3 years (49%) followed by the 4-6 years group (20%) then the 7-9 years group then less than 1(4%) before finally the 10 years and above at 2%.

The association of sex of the host and the prevalence of *E. canis* infection was non-significant ($p=0.767$). A significant association between the prevalence of *E. canis* infection and the breed of the dog was observed ($p=0.019$). The host's age was also found to be statistically significant in association with the prevalence of *E. canis* infection ($p=0.001$) in relation to serological test.

4.5 *Ehrlichia* Culture Test Analysis

Plasma of 20 dogs (20%) were positive for *Ehrlichia* culture test as demonstrated by aggregation of intracytoplasmic initial inclusion bodies/organisms staining purple in colour (Plates V and VI). The 2 dogs that revealed intracytoplasmic organisms in their plasma were also observed on

buffy coat smears and the 20 dogs were also positive for the serological test; 18 dogs that did not reveal organisms in buffy coat smears showed positive indications using the culture medium.

The results from the culture analysis were also analyzed in respect of breed, age and sex predispositions. Analysis of results with respect to sex (Table 3) showed a higher prevalence rate in males (12%) than females (8%). No significant association was shown between sex of the host and culture test with a p-value of 0.837. The age related prevalence of *E. canis* infection (Table 4) and *Ehrlichia* Culture showed the highest prevalence in ages 1-3 (10%) followed by ages 4-6 (7%) then, less than 1 year (2%) and finally 7-9 years (1%). No prevalence rate was recorded in ages 10 and above. There was no significant association between *Ehrlichia* Culture and age of the host (p=0.544). The breed specific prevalence of *E. canis* infection (Table 5) showed the highest rate in Local dogs (16%) compared with the Cross breeds (4%). The Exotic breeds had a 0% prevalence rate. There was no significant association seen between *Ehrlichia* Culture and breed related prevalence of *E. canis* infection.

4.6 Haematological Analysis

The most prominent feature observed in the study was thrombocytopenia (Table 8) as all the dogs (100%) revealed values below normal reference range (211-621 x 10^9L) for both the positive and negative groups respectively. There was also a significant difference of $p \leq 0.05$ for thrombocytes.

Evaluating the haematological parameters using *Ehrlichia* culture test showed a significant difference between the neutrophils (60.10±5.11 & 51.23±2.24), basophils (2.45±0.87 & 0.54±0.16) and band cells (0.15±0.11 & 0.58±0.14) for both positive and negative groups

respectively. The eosinophils, basophils and band cells were seen to have values within reference range (0-1%, 0-9% and 0-3% respectively) while neutrophils (58-85%) showed a slight increase in the negative group and were normal for the positive group. PCV, Hb, WBC, RBC, TP, lymphocytes, monocytes and eosinophils showed no significant difference. However, RBC, TP and lymphocytes indicated increased values in the positive group and also increased values for the negative group for TP and lymphocytes while the RBC values were normal.

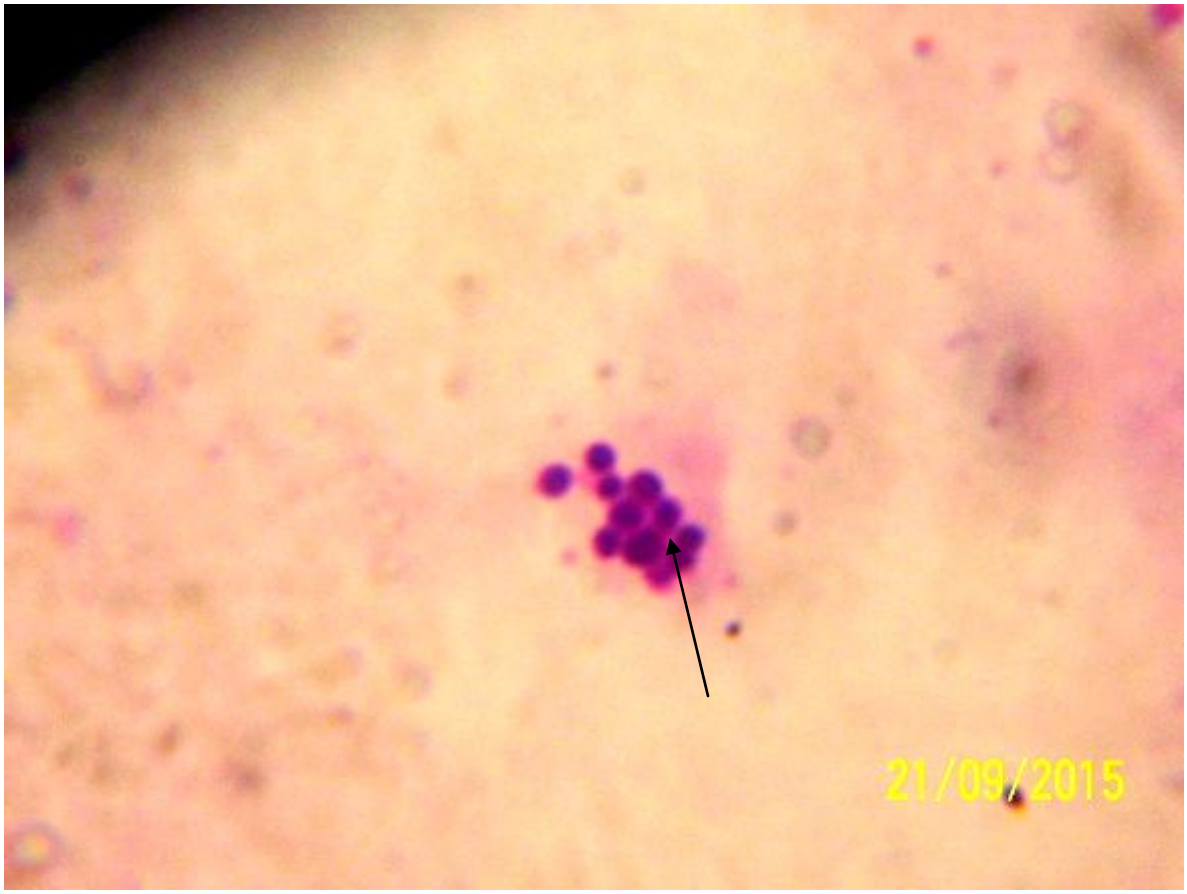

Plate V: Initial bodies of *Ehrlichia canis* (black arrow) in plasma of infected dog using *Ehrlichia* culture.

Magnification = x5000 (100 objective x 10 eyepiece x 5x optical zoom)

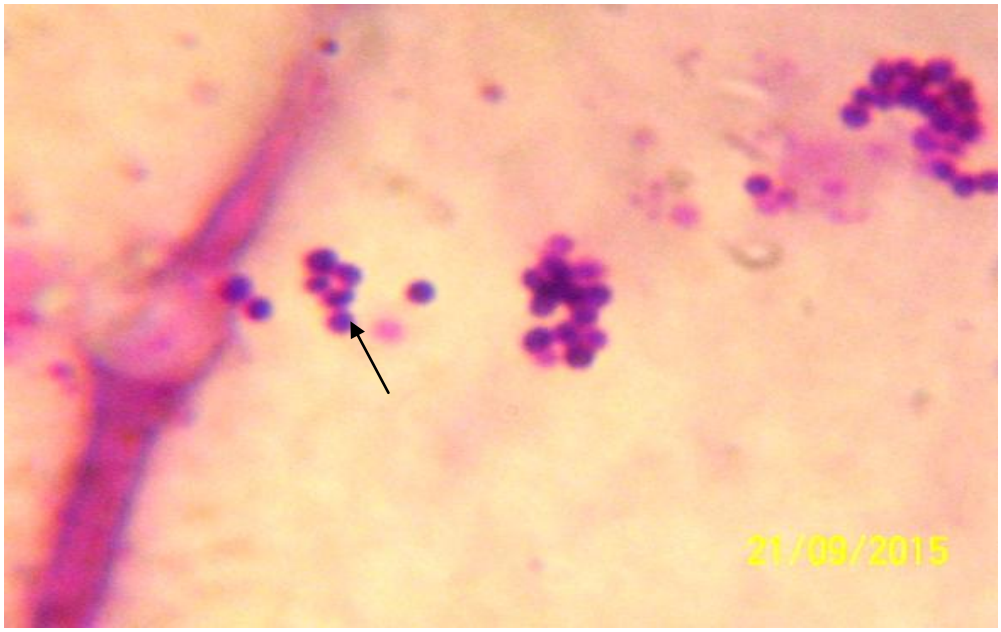

Plate VI: Initial bodies of *Ehrlichia canis* (black arrow) in plasma of infected dog using *Ehrlichia* culture.

Magnification = x5000 (100 objective x 10 eyepiece x 5x optical zoom)

Table 4.8: Haematological parameters of dogs detected with *Ehrlichia canis* infection using *Ehrlichia* culture test

Haematological parameters	Means ± SEM Positive n = 20	 Negative n = 80
PCV (%)	49.94 ± 3.86	48.56 ± 1.31
Haemoglobin (g/dL)	16.20 ± 1.19	15.32 ± 0.48
WBC (10^9/L)	10.46 ± 1.07	9.92 ± 0.47
RBC (10^6/µL)	8.15 ± 0.58	7.63 ± 0.20
Total proteins (g/dL)	8.00 ± 0.39	7.65 ± 0.18
Neutrophils (%)	60.10 ± 5.11[a]	51.23 ± 2.24[b]
Lymphocytes (%)	36.65 ± 4.97	46.03 ± 2.16
Monocytes (%)	0.65 ± 0.29	1.74 ± 0.52
Basophils (%)	2.45 ± 0.87[a]	0.54 ± 0.16[b]
Eosinophils (%)	0.00 ± 0.00	0.00 ± 0.00
Band cells (%)	0.15 ± 0.11[a]	0.58 ± 0.14[b]
Thrombocytes (10^9/L)	100.15 ± 11.00[a]	123.63 ± 11.09[b]

Superscripts a, b: means with different letters in the same rows are significantly different at $p \leq 0.05$

4.7 Biochemical Profile

Sera samples from the same lot of 100 dogs were subjected to estimation of enzymes and other functions that denote liver and kidney functions.

For serum biochemical parameters in relation to *Ehrlichia* culture test (Table 9), albumin (36.42±5.28 & 28.91±2.32), ALT (9.47±1.61 & 12.9±1.61), urea (3.20±0.49 & 3.34±0.26) and creatinine (52.95±6.96 & 56.27±3.19) had no significant difference for both the positive and negative groups respectively. TP (76.32±9.44 & 55.97±3.76), ALP (79.89±13.56 & 119.05±13.34) and globulin (39.95±5.78 & 27.62± 2.14) had significant differences where $p \leq 0.05$ for both the positive and negative groups respectively. Urea and creatinine were all within reference ranges (2.9-10mg/dL and 44-150mg/dL) in both groups. TP, globulin and albumin levels were elevated in the positive group however, albumin was normal in the negative group while TP was decreased and globulin slightly increased. ALP levels were normal in the positive group and elevated in the negative group.

Table 4.9: Serum biochemical parameters of dogs detected with *Ehrlichia canis* infection using *Ehrlichia* culture test

	Means ± SEM	
Serum biochemical parameters	**Positive n = 20**	**Negative n = 80**
Total proteins	76.32 ± 9.44[a]	55.97 ± 3.76[b]
Albumin	36.42 ± 5.28	28.91 ± 2.32
ALT	9.47 ± 1.61	12.9 ± 1.61
ALP	79.89 ± 13.56[a]	119.05 ± 13.34[b]
Globulin	39.95 ± 5.78[a]	27.62 ± 2.14[b]
Urea	3.20 ± 0.49	3.34 ± 0.26
Creatinine	52.95 ± 6.96	56.27 ± 3.19

Superscripts a, b: means with different letters in the same rows are significantly different at $p \leq 0.05$; ALT = Alanine aminotransferase; ALP = Alkaline phosphatase

CHAPTER FIVE

5.0 DISCUSSION

Canine ehrlichiosis, a common disease entity in dogs in Nigeria is tentatively diagnosed on the basis of clinical signs of fever, splenomegaly, peripheral lymphadenopathy, depressed appetite, depression, haemorrhages on visible mucosal surfaces and bleeding tendency (Hassan *et al*., 1990). Although dogs in this study appeared apparently healthy, accidental findings like fever, blood tinged urine and presence of ticks were observed. However, these clinical signs might also be seen in other infectious diseases like leptospirosis, babesiosis e.t.c and hence, differential diagnosis is essential for proper curative and prophylactic treatment. Presently, in most of the laboratories in Nigeria, emphasis is given on either the demonstration of the organisms in blood or buffy coat smears. However, both techniques have their own limitations which pose a serious problem for clinicians. Clinical and laboratory diagnosis of *E. canis* infection is a challenging task, since the disease presents with varied clinical, haematological, as well as biochemical abnormalities (Waner, 2008). Apart from the conventional parasitological diagnostic techniques therefore, cultures and serological assays are now also increasingly utilized for the detection of the parasite.

In this study, the morphologic features of the parasite matched the description of *E.canis* (Bool *et al*., 1957; Philip, 1957, McDade, 1990; Paddock *et al.,* 2003). However, the level of parasitaemia was low except with culture test which recorded 20% detection rate. Similar observations of low detection rate and low level of parasitaemia were also reported by Joshua *et al*. (2013); Woody and Hoskins (1991); Juyal *et al*. (1994); Thriunavukkarasu *et al*. (1993); Waner *et al*. (1999b) and Nakaghi *et al*. (2008) in spite of exhibition of typical signs of the disease. The former

authors reported as low as 0.2% parasitaemia in dogs (Inokuma *et al*., 1999) infected with *E. canis*. On the other hand, Lawal *et al,* (2001); Katyal (2000); Lakshmanan (2001); Mallapur (2002); Samradhni *et al*. (2003) reported higher detection rates of 6.8%, 17.58%, 55%, 18.9% and 5.8% respectively. This discrepancy in the detection of the rickettsia might be due to the clinical phase of the infection as demonstrated by tenacity of laboratory personnel to inspect the large number of cells in each smear. The lower prevalence recorded by blood smear examination is due to the fact that it is less sensitive (Woody and Hoskins, 1991; Stich *et al*., 2002 and Nakaghi *et al*., 2008). Also, the chances of successfully finding the morulae in stained smears are very low, particularly in the subclinical phase of the disease (Harrus and Waner, 2010) as it is assumed that during this phase, the pathogen is sequestered within the spleen and hence, is not detected in blood (Waner, 2008). Further, the inability of differentiating morulae from the inclusions present during severe bacterial infections, inflammations, auto-immune diseases, viral infections and severe tissue destruction may result in false positive results.

It was also noted by earlier studies that the prevalence (Katyal, 2000; Hassan *et al*., 1990) as well as the severity of clinical signs (Katyal, 2000; Huxsoll *et al*., 1970, Schillhorn van Veen and Adeyanju, 1979) was higher in German Shepherds. In another study, Thriunavukkarasu *et al*., (1994) and Lakshmanan (2001), noted higher prevalences in pure breed dogs as compared to the local breeds. This discrepancy might be due to a number of factors like nutritional, immunological competence and susceptibility of different breeds (which breed) to tick infestation (Ashuma *et al*., 2005). In this study, we found the detection of *E. canis* infection to be higher in the local (indigenious breeds) than the exotic breeds. This is in agreement with work done by Hassan *et al*. (1990) and it was assumed that this could be due to their being generally poorly cared for and nutrition when compared to exotic breeds or their crosses. Exotic breeds are

usually more cared for by their owners. The prevalence of *E. canis* infection was higher in males than in females in this study. This can be attributed to territorial habits associated with males, as they roam more widely than the females. This finding is in agreement with work done by Hassan *et al*. (1992). However this was not true for other works of Lakshmanan (2001) and Milanjeet *et al*. (2014) showing a reverse trend. There was also no age or sex association with CE as earlier reported by Price and Sayer (1986); Huxsoll *et al*. (1972); Tresamol *et al*. (1998); Rahman *et al*. (2010). In this study however, age-related prevalence was recorded to be higher in dogs aged 1-3 years. This agrees with work done by Chetari *et al*. (2012). Other studies show a higher prevalence in dogs less than a year as seen in Bhadesiya and Raval (2015) and Hassan *et al*. (1992). Several authors have found the prevalence of tick-borne infections to be highest in young dogs than adults (Ezekoli *et al*., 1983; Abdullahi *et al*., 1990, Samaradni *et al*., 2003; Lakshmanan *et al*., 2006). This increased probability could be due to the naivety of their immune status. It is shown in this study that age has a significant effect on the occurrence of *E. canis* infection consistent with earlier findings.

Rapid Anigen® antibody test (immunochromatographic assay) does not diagnose early infections and cannot differentiate current and past infections. The disparity observed between the two diagnostic tools viz, conventional (blood smears) and immunodiagnostic methods, (serology) may be due to low levels of parasitaemia leading to detection of less number of cases. Nevertheless, higher detection rate elicited by the rapid antibody test in the present study could be attributed to failure of the assay to differentiate current and past infections (Waner *et al*., 2007 and McBride *et al*., 2001). One more drawback reported to be associated with immunodiagnostic methods is failure of the tool to detect early infections (McBride *et al*., 2001). The immunodiagnostic methods, though user friendly and quick, may not detect early infections due

to inadequate immunostimulation leading to false negative results and may not rule out past infections due to persistence of antibodies in the circulation leading to false positive results.

A slight level of agreement was seen between the various diagnostic tests. This could be attributed to the wide variations in detection rates or values.

Profound haematological changes have been found to occur during acute, subclinical and chronic phases of canine ehrlichiosis (Harrus *et al*., 1999 and Waner, 2008). All the dogs included in this study showed thrombocytopaenia. However, thrombocytopaenia, an important haematological finding in CE does not rule out other diseases like leptospirosis. Lowered blood counts or thrombocytopaenia are often critical signs of ehrlichial infection. In a study conducted by Shipov *et al*. (2008), red blood cell (RBC) count and Haemoglobin (Hb) were used as the diagnostic indicators for canine ehrlichiosis. Further, dogs negative for *E. canis* infection in the present study also exhibited a lower platelet count. It has been observed that not all thrombocytopaenic dogs are positive for *E. canis* infection, suggesting that it is not the only cause of thrombocytopaenia in dogs (Dagnone *et al*., 2003; Trapp et al., 2006; Gaunt *et al*., 2010). Numerous diseases can result in thrombocytopaenia including immune mediated thrombocytopaenia, neoplastic processes, inflammatory diseases or other infectious agents (Grindem *et al.*, 2002). Smith *et al*., (1997) suggested that thrombocytopaenia in *E. canis* infection is mainly due to large scale destruction of cells in the spleen that begins within few days after the infection but Waner (2008) pointed out that the bone marrow hypoplasia leading to impairment of normal functions is the primary cause of pancytopaenia including thrombocytopaenia.The development of thrombocytopaenia has also been attributed to an immunopathological mechanism by Waner *et al*., (1999b) who demonstrated that significant levels of serum antiplatelet IgG, 17 days after experimental *Ehrlichia canis* infection that

resulted in the removal of antibody-absorbed thrombocytes by the mononuclear phagocyte system in the liver and spleen; involvement of non-immunological mechanisms may also contribute in aggravating the condition. Decreased Hb (Heamoglobin), Packed Cell Volume (PCV) could be due to epistaxis, petechial haemorrhages, myelosuppression or due to severe anaemia (Bhardvaj, 2013). This was not the same as in our study as Hb and PCV values were found within reference ranges and of no significant difference. This could be attributed to several influential factors such as nutritional status, iron reserves in the body, stage of the infection, concurrent infections and the age of the infected dogs. Among diffferntial leukocyte count, levels of lymphocytes increased significantly. This finding was in agreement with Dixit *et al*., (2012). However, Castro *et al*., (2004) and Oliveria, (2000) observed lymphocytopaenia associated with ehrlichiosis. Levels of monocytes decreased significantly in the cases for ehrlichiosis as in agreement with Oliveria (2000). However, Castro *et al*., (2004) reported monocytosis.

Among various serological parameters, the levels of total protein increased significantly in dogs serologically positive for *E. canis* than in the negative ones, which are in agreement with Bhardwaj, (2013); Irwin, (2007) and Weiser *et al*., (1991). On the other hand, hypoproteinaemia was seen in reports by Castro *et al*., (2004); Srikala *et al*., (2012); Srivastava and Srivastava, (2011); Agnihotri *et al*., (2012) and Harrus *et al*., (2004). Levels of ALP and ALT were found to be normal except in one case where there was decreased ALT. Creatinine and urea were found to be within reference ranges in all other groups.

5.1 CONCLUSION

- Serology using the rapid antibody test was more sensitive (83%) than the conventional blood smear examination and *Ehrlichia* culture test in detecting *E. canis* in dogs in this study area. This appeared to be logical owing to extremely low levels of parasitaemia in blood smears and the the inability of the serological test to differentiate ongoing and past infections.
- *Ehrlichia* culture test was more sensitive (20%) than the routine blood smears (2%).
- Physical examination revealed that animals appeared apparently healthy, but some presented signs as fever, pale mucus membrane and emaciation.
- A high prevalence of CE was observed in males (62%) than females (38%) and the highest prevalence was observed in young animals aged 1-3 years.
- The indigenious breed of dogs had a high prevalence rate of 77% as compared to the exotic breed.
- Basawa ward had a low prevalence of 5% using the serology while Dogarawa revealed a prevalence of 40% using *Ehrlichia* culture test.
- There was a slight level of agreement between the applied diagnostic methods (buffy coat smear vs *Ehrlichia* culture; 0.151; $p< 0.04$ and buffy coat smear vs serology; 0.008; $p< 0.518$). However, there was a significant association between buffy coat smear and *Ehrlichia* culture test ($p= 0.04$).
- There was a slight level of agreement between serology and *Ehrlichia* culture test (0.069; $p< 0.110$).
- Haematological analysis revealed thrombocytopenia as the most consistent finding in all the examined dogs.

- Lymphocytosis and lowered monocytes were also prominent in both positive and negative groups of dogs.
- Serum biochemical analysis revealed hyperproteinaemia as the most prominent finding in the positive group of dogs.

5.2 RECOMMENDATIONS

- Ehancement of the *Ehrlichia* culture test to be used routinely in the laboratories and for clinical diagnosis. This should be used especially for high suspect cases.
- A combination of parasitological and serological methods are recommended for rapid detection of *E. canis* infection in dogs.
- Repeated buffy coat smear examinations should be conducted to improve the efficacy of the diagnosis.

5.3 LIMITATIONS OF STUDY

- Unwillingness of clients to enroll their dogs for sampling especially owners of exotic dogs.
- The rapid serological test kits are costly and not readily available.
- Time consumption, tedious steps and power outages limit the routine use of *Ehrlichia* culture test.

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APPENDICES

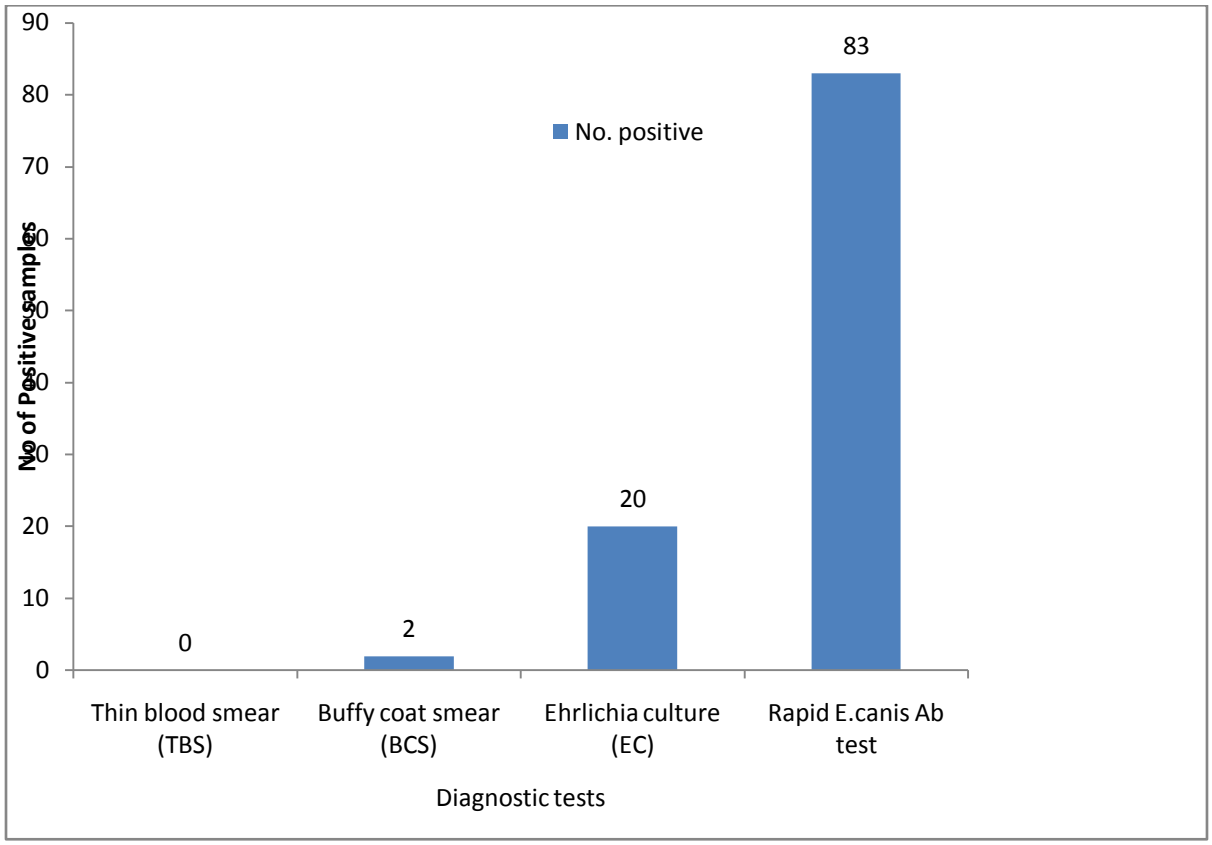

Figure 2: Prevalence of *Ehrlichia canis* infection using various diagnostic tests .

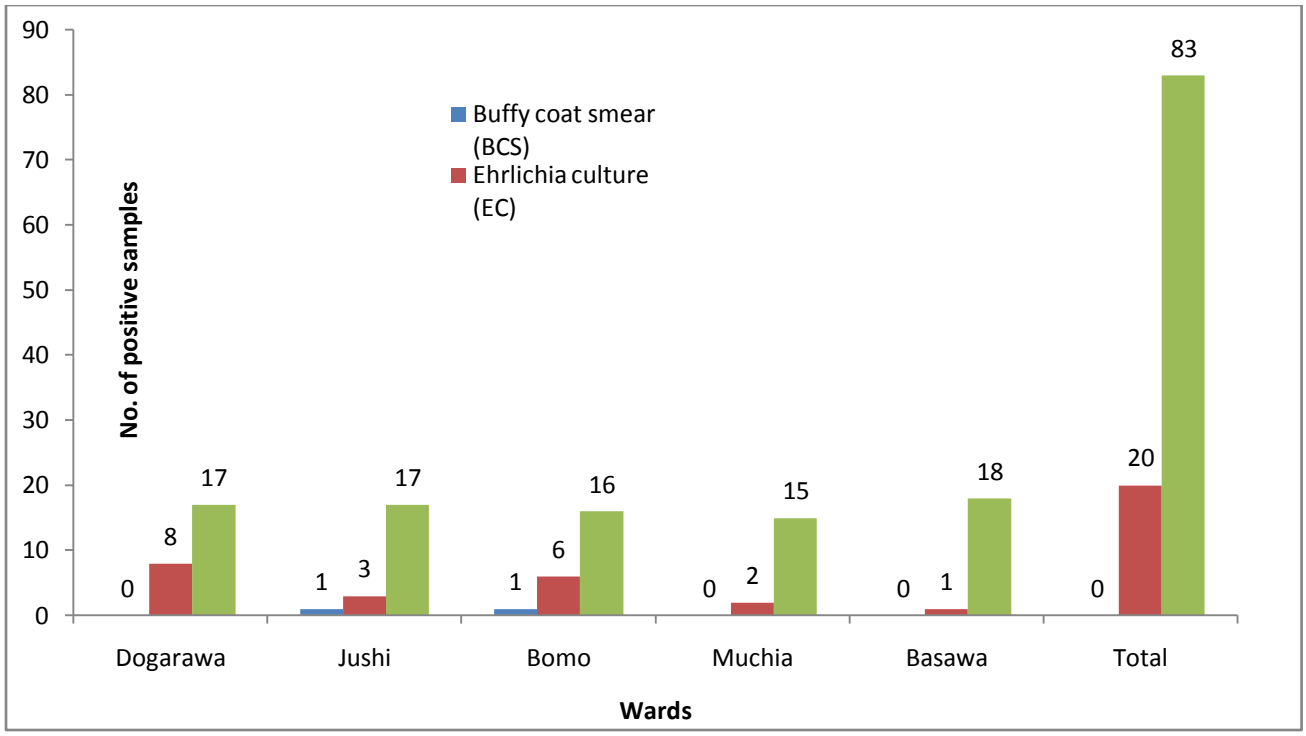

Figure 3: Prevalence of *Ehrlichia canis* infection in the selected wards using the different diagnostic techniques

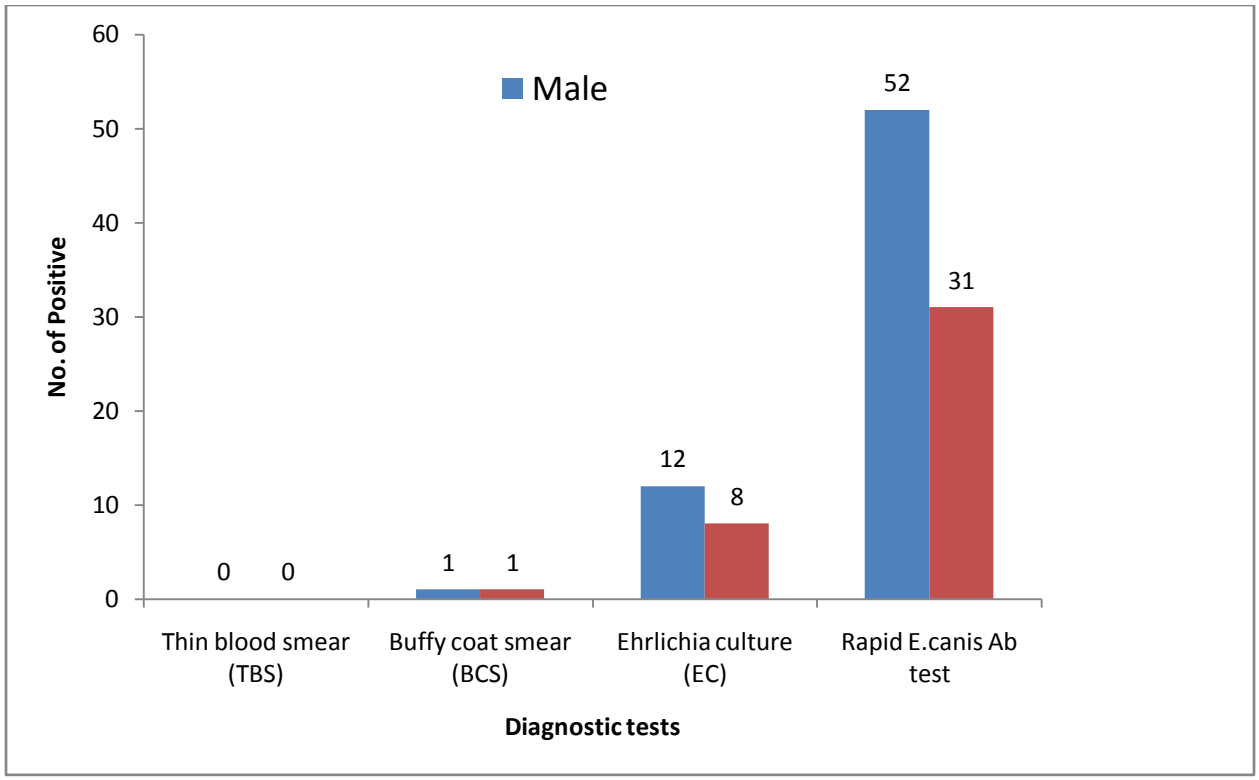

Figure 4: Sex related prevalence of *Ehrlichia canis* infection across tests

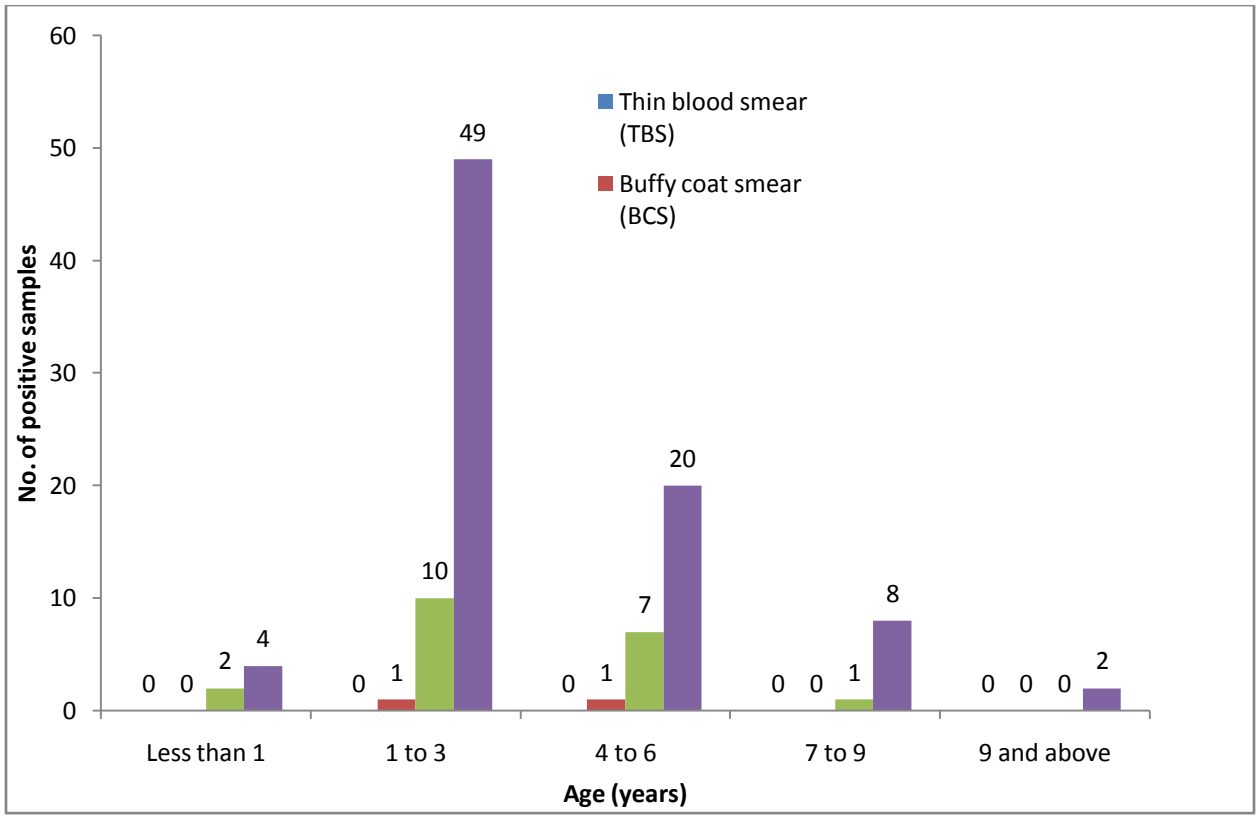

Figure 5: Age related prevalence of *Ehrlichia canis* infection across tests

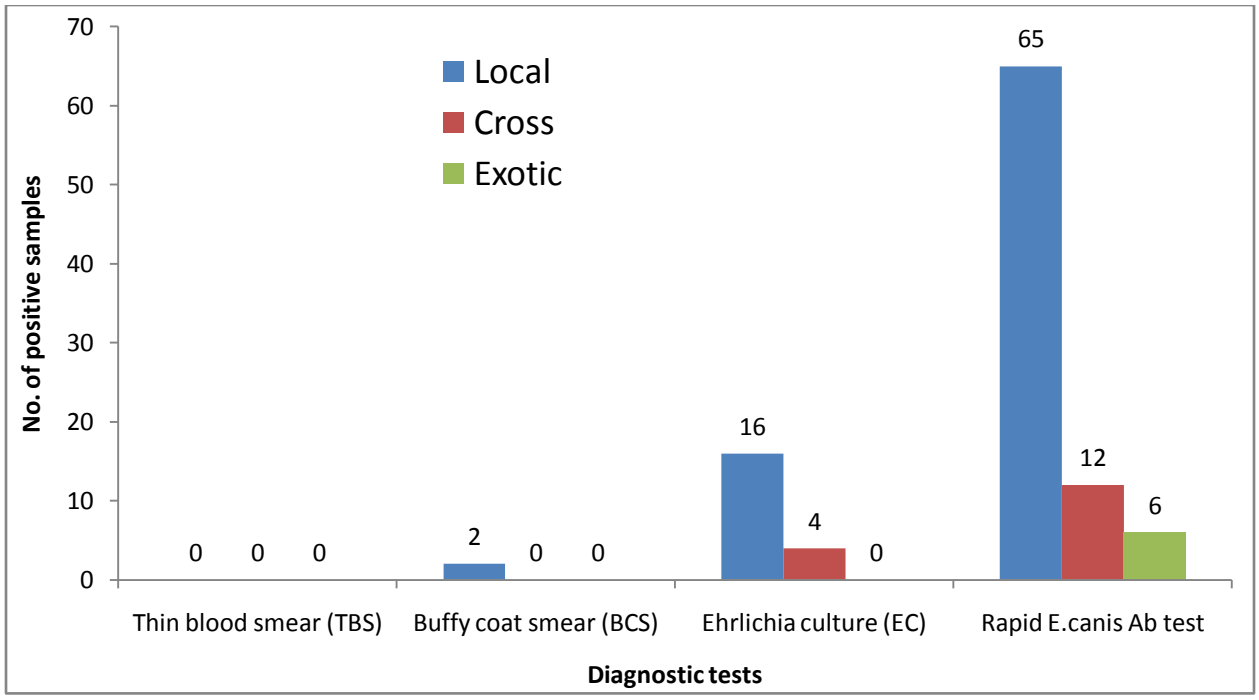

Figure 6: Breed related prevalence of *Ehrlichia canis* infection across tests

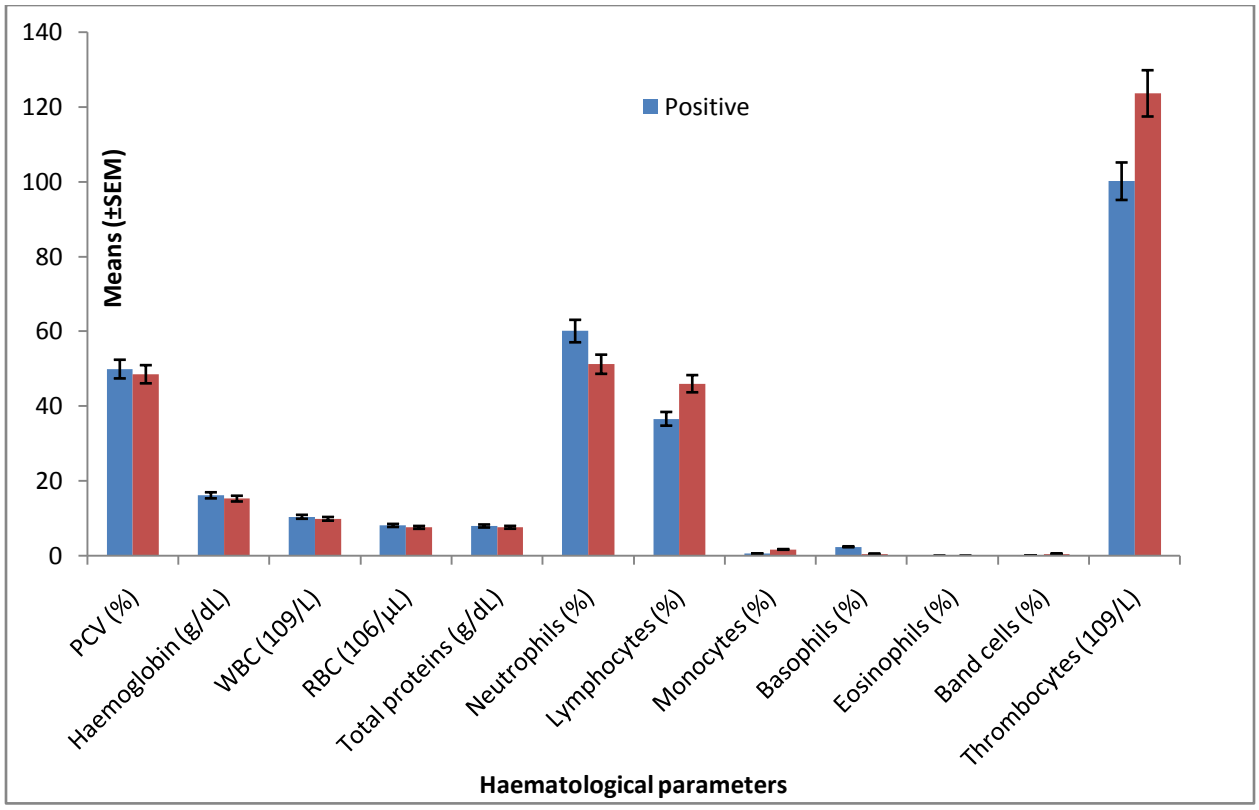

Figure 7: Haematological parameters of dogs with *Ehrlichia canis* infection using *Ehrlichia* culture test

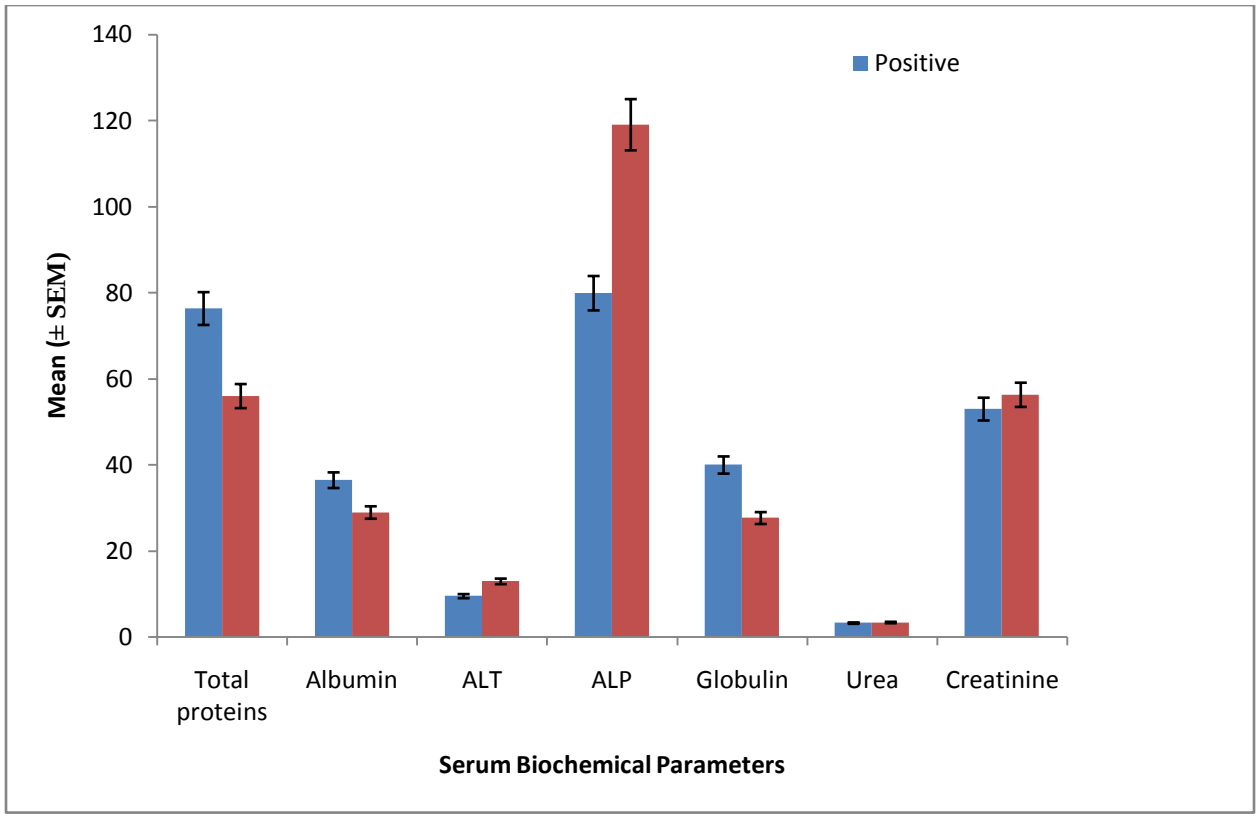

Figure 8: Serum biochemical parameters dogs with *Ehrlichia canis* infection using *Ehrlichia* culture test