CLINICAL, HAEMATOLOGICAL, TREATMENT AND MOLECULAR EVALUATION OF EHRLICHIAL INFECTIONS IN DOGS IN NAIROBI AND ITS ENVIRONS

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A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN CLINICAL STUDIES OF THE UNIVERSITY OF NAIROBI

DEPARTMENT OF CLINICAL STUDIES
FACULTY OF VETERINARY MEDICINE
UNIVERSITY OF NAIROBI

November 2014

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university

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DEDICATION

To

My spouses Edith Jumba and Maryanne Imbenzi and our children.

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ACRONYMS

HR: Heart rate

RR: Respiratory rate

TEMP: Temperature

WBC: White blood cells

LYMP: Lymphocytes

LYMPHM: Lymphocyte mature

MONO: Monocytes

MONOCYM: Monocytes mature

GRANUL: Granulocytes

NEUTRO: Neutrophils

NEUTROM: Neutrophil mature

EOSINO: Eosinophils

BASO: Basophils

RBC: Red blood cells

MCV: Mean corpuscular volume

HCT: Haematocrit

MCH: Mean corpuscular haemoglobin

MCHC: Mean corpuscular haemoglobin concentration

RDW: Red Cell Distribution Width

HB: Haemoglobin

THROMBO: Thrombocytes

MPV: Mean platelet volume

PCT: Platelet crit

PDW: Platelet Distribution Width

BUN: Blood urea nitrogen

TP: Total protein

A/GRATIO: Albumin/globulin ratio

AP: Alkaline phosphatise

ALT: Alanine aminotransferase

ACKNOWLEDGEMENT

ABSTRACT

Ehrlichiosis and Anaplasmosis are tick borne diseases with important zoonotic and public health implications. Although reported globally, data on apparent prevalence, clinical, haematological and treatment outcomes and molecular characteristics of ehrlichial infections in dogs in Kenya is scant.

A study was therefore designed with the objectives to retrospectively determine the clinical features and treatment of ehrlichiosis in dogs; to evaluate clinical, haematological and biochemical features and treatment outcomes in dogs with ehrlichiosis; and to determine the molecular profiles and apparent prevalence of canine ehrlichiosis in Kenya. The retrospective study component entailed review of clinical and treatment records of 514 dogs diagnosed with ehrlichiosis at the Small Animal Clinic (SAC), Faculty of Veterinary Medicine, University of Nairobi between 1993 and 2006. The prospective study comprised evaluation of clinical, haematological, biochemical and treatment data on ehrlichial infections in dogs presented at the SAC. Identification of the ehrlichial species was performed using Polymerase Chain Reaction (PCR) with genus-specific and species specific primers based on ehrlichial 16S rRNA genes and sequence analyses. Data were analysed using descriptive statistics and independent t-test at a confidence interval of 95% (p≤0.05).

Males were over-represented at 54.4% among the 479 dogs diagnosed with ehrlichiosis, with a large proportion of affected breed being the German shepherd dog (42.6 %). Clinical findings included lethargy, anorexia, fever, panting, lymphadenomegaly, pallor and congestion of mucous membranes, pounding heart, harsh lungs, vomiting, tender

abdomen, splenomegaly, ocular discharge, hind limb weakness, dermatitis, diarrhoea and haemorrhage. Lymphadenomegaly was the most commonly observed clinical sign in dogs suffering from ehrlichiosis and was observed in 65.3% and in 59.3 % of cases in the prospective and retrospective studies respectively. Congestion of mucous membranes was the most frequently observed sign and was reported in 55.4% and 48.7% of the dogs diagnosed with ehrlichiosis in the prospective and retrospective studies respectively. Pale mucous membranes were observed in 16.4% and 21.3% of the cases in the prospective and retrospective studies respectively.

Haematological and biochemical parameters were significantly increased ($p<0.05$) (mean lymphocyte counts, and haematocrit). Although erythrocyte and thrombocyte counts and mean haemoglobin concentration were increased, these were not statistically significant. The mean granulocyte counts decreased significantly ($p<0.05$) following diagnosis and 14 days post-treatment. Significant ($p<0.05$) increases were observed in serum albumin and calcium levels in dogs diagnosed with ehrlichiosis in this study.

Treatment resulted in clinical improvement, noted by resolution of anaemia, and altered haematological parameters, including significantly elevated Packed Cell Volume (PCV) ($p<0.05$). Congestion of mucous membranes reduced from 55.4% to 27.7%. Imidocarb dipropionate was the most preferred treatment (82.6 %), compared to Doxycycline (2.8 %) or a combination of the two drugs (14 %). Although 49.1% of treated dogs reportedly recovered, 43.5% were lost to follow up 14 days post-treatment. Recovery following treatment with Imidocarb dipropionate was very high, as only 7.3% of dogs did not show clinical improvement.

Molecular analysis revealed 58.6% (113/192) of dogs had positive amplification of ehrlichial DNA by PCR using primers ECC and ECB that amplify a sequence of the 16S rRNA gene of Ehrlichia and Anaplasma genera. Of the positive cases, 42.5% (48/113) were positive for *Ehrlichia canis* using the species specific primers HE3 and ECANS5, 5.3% (6/113) were positive for *Ehrlichia chaffeensis* with the species specific primers HE1 and HE3 and 1.8% (2/113) was positive for *Anaplasma platys* with the species specific primers PLATYS and EHR16SR.The most consistently observed clinical features in *Ehrlichia canis* infected dogs were lymphadenopathy, congestion of mucous membranes, inappetence, panting, loose hair, lethargy, vomiting, wasting, ocular discharge and diarrhoea. Signs for *Ehrlichia chaffeensis* infection included lethargy, lymphadenopathy, congestion of mucous membranes and inappetence; these were consistently present in affected dogs. On the other hand, signs of *Anaplasma platys* infection included lethargy, lymphadenopathy, ocular discharge, inappetence, panting, pounding heart, harsh chest, loose hair and wasting.

The most commonly observed clinical signs in ehrlichiosis and anaplasmosis included lethargy, lymphadenopathy, pallor and congestion of mucous membranes, inappetence, loose hair, wasting, ocular discharge, harsh lungs and panting. Congestion of mucous membranes, lymphadenopathy, lethargy and inappetence were most consistently observed in dogs with ehrlichiosis.

The study identified two additional ehrlichial species; *Ehrlichia chaffeensis* and *Anaplasma platys,* in Kenya, previously not reported. The study has also established the molecular identity of *Ehrlichia canis*, *Ehrlichia chaffeensis* and *Anaplasma platys* in dogs in Kenya, previously not reported. These findings confirm the zoonotic importance and

public health implications of canine ehrlichiosis. Further investigations are recommended to determine the molecular epidemiology of ehrlichial infections in animals and humans in the region. It is also noted that congestion of mucous membranes is an important clinical sign in canine ehrlichiosis. The clinical presentation is not specific but congestion of mucous membranes, lymphadenopathy, lethargy and a history of inappetence coupled with thrombocytopenia form good inclusion criteria for a tentative diagnosis of ehrlichia infection where diagnostic services are limited.

CHAPTER 1

1. Introduction and Objectives

1.1 Introduction

Ehrlichiosis and anaplasmosis are globally recognized as important tick borne infectious diseases of dogs and other canids, with higher frequency in tropical and sub-tropical regions (Ravyn *et al*. 1999; Suto *et al*. 2001; Alexandre *et al*. 2009).

Ehrlichiosis is caused by obligatory intracellular gram-negative bacteria belonging to the family *Anaplasmataceae* genera *Ehrlichia* and *Anaplasma*, which infect monocytes, granulocytes, and platelets (Harrus *et al*. 1998). Within the mammalian hosts, ehrlichial organisms demonstrate tropism for leukocytes and multiply within endosomes, producing cytoplasmic inclusions called morulae which do not fuse with lysosomes (Sumner *et al*. 2000). *Anaplasma phagocytophilum* targets and replicates within neutrophil granulocytes (Woldehiwet *et al*. 2006; Carlyon and Fikrig, 2003).

Molecular and antigenic analyses have been used to segregate Ehrlichia species into three monophyletic clades that were commonly referred to in ehrlichial literature as genogroups and that bear names of the prototype species, *Ehrlichia canis, Ehrlichia phagocytophila, and Ehrlichia sennetsu* (Sumner *et. al*. 2000). However, based on the degree of similarity of the 16S rRNA genes of the species, Dumler *et al.* (2001) proposed a new classification of these pathogens. These are *Anaplasma phagocytophilum* which joined together three previously described species *Ehrlichia equi, Ehrlichia*

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phagocytophilum, and human granulocytic agent (HGE agent). *Ehrlichia sennetsu* is reclassified as *Neorickettsia sennetsu*. *Ehrlichia canis*, *Ehrlichia ewingii* and *Ehrlichia chaffeensis* retained their classification.

The organisms are transmitted by various species of ticks such as the brown dog tick, *Rhipicephalus sanguineus*, which is endemic world-wide, *Dermacentor variabilis*, *Amblyomma americanum*, *Ixodes persulcatus, Ixodes scapularis* and *Ixodes pacificus*. *Anaplasma phagocytophilum* has been identified in *Haemaphysalis longicornis* ticks (Kim *et al*. 2003).

High *E. canis* seroprevalence rates have been reported among dogs in North America, Europe, the Middle East, North and South Africa. However, information on the prevalence of *E. canis* among dogs detected by molecular techniques such as PCR is scanty. A study by Kordick *et al*. (1999) reported 56% of dogs in a kennel in North Carolina were *E. canis* positive as determined by PCR. Murphy, *et al*. (1998) detected *E. canis* DNA in 3% of dogs in Oklahoma. Unver *et al*. (2001b), reported 31% of Venezuelan dogs were positive by PCR specific to *E. canis*. The high infection rate shows that *E. canis* is a pathogen among the dog population in these locations, and requires attention by veterinarians and public health professionals. Molecular typing of the etiological agents for ehrlichial infection in Kenya has not been carried out and is warranted to further elucidate the infections in companion animals and their zoonotic potential.

Human ehrlichioses are tick-borne illnesses of worldwide importance (Pritt *et al*. 2011; Ismail *et al*. 2010; Ravyn *et al*. 1999; Perez *et al*. 1996). Three Ehrlichia species have

been recognized as human pathogens transmitted by ticks in the United States (Sumner *et al*. 2000). It has been proposed that humans are at a risk of being infected with this Ehrlichia species when a tick, most likely *Amblyomma americanum*, bites both animals and humans. *Anaplasma phagocytophilum* a causative agent of tick-borne fever in small ruminants is also the agent for human granulocytic anaplasmosis (Stuen 2007; Woldehiwet *et al*. 2006). However, no scientific reports are available describing human ehrlichiosis in Kenya to date.

A disease in dogs resembling ehrlichiosis was first reported in and around Nairobi, Kenya by Danks (1937) and later by Murray (1968). The etiological agent was later identified as *Ehrlichia canis* using cell culture isolation and indirect fluorescent antibody tests (Kaminjolo *et al*. 1976) and using serology in free ranging jackals in Kenya (Alexander *et al*. 1994).

Price (1980) described clinical and haematological features of natural and experimental canine ehrlichiosis. However, no studies have been conducted on the molecular characterization of the infective agent or to confirm the identity of the etiologic agent causing ehrlichiosis. A single report has described feline ehrlichiosis in Kenya (Buoro *et al.* 1986), but the species involved was not identified.

Seroprevalence studies have suggested the presence of *Ehrlichia canis* or related species infecting dogs throughout Africa (Pretorius and Kelly, 1998; Brouqui *et al*. 1991; Bostros *et al*. 1995; Kelly *et al*., 2004). High titres were noted for *Ehrlichia chaffeensis* than to *Ehrlichia canis* in 7 dogs from South Africa, an indication that *Ehrlichia chaffeensis* was the probable etiologic agent in those cases (Pretorius and Kelly 1998). Studies by Ndip *et*

al. (2005) in Cameroonian dogs reported the presence of *Ehrlichia canis* and *Ehrlichia ewingii* by PCR. It is therefore necessary to investigate and establish which species of these ehrlichial organisms are present in Kenya.

Although canine ehrlichiosis is a common clinical entity in Kenya, scientific data on molecular identity and prevalence of the ehrlichial species causing disease is scant. Such information would elucidate the full extent of its clinical features and assist in refining the case definition for each type of ehrlichial infection and further aid in tentative diagnosis, particularly where confirmatory diagnostic tools are not readily available.

1.2 Objectives

1.2.1 Overall objective

To provide information and knowledge on clinical, haematological, molecular characteristics, treatment and response of ehrlichial infections to refine the clinical case definition and support tentative diagnosis or for surveillance studies in Kenya.

1.2.2 Specific objectives

The specific objectives of the study were;

1. To determine the clinical presentation and treatment outcomes in dogs diagnosed with ehrlichiosis at the Small Animal Clinic, Faculty of Veterinary Medicine, University of Nairobi between 1993 and 2006.

2. To evaluate the clinical, haematological, biochemical features and treatment outcomes of natural ehrlichial infections in dogs.

3. To determine using Polymerase Chain Reaction (PCR) the ehrlichial species and their apparent prevalence in natural infections in dogs in Kenya.

1.3. Justification

Ehrlichiosis has been diagnosed in Kenya by researchers who confirmed the causative agent by cell culture and serological techniques. However, studies describing molecular characteristics of the ehrlichial species causing the disease in Kenya have not been reported in scientific publications. Furthermore, evidence of molecular identity of the species of ehrlichial organisms circulating in the canine population in Kenya is scant. Studies on the apparent prevalence, clinical, haematological and molecular diagnosis of ehrlichial infection are warranted. The information on the species range in the canine population is important as some of the ehrlichial species are known to be zoonotic hence of great public health concern.

CHAPTER 2

2. General Literature Review

2.1   Definition of ehrlichial disease

Ehrlichiosis is a potentially fatal tick borne disease of dogs. Canine ehrlichiosis is a multisystemic disorder, and generally appears as an acute disease with varying clinical signs (Egenvall *et al*. 1997). Though naturally acquired feline infection has been documented for over a decade, researchers are only beginning to elucidate the relevance of ehrlichiosis in cats. In dogs, the agents can cause acute, sub clinical or chronic disease. It is reported worldwide due to the association with the broad distribution of its vector, *Rhipicephalus sanguineous* (Stubbs *et al*. 2000; Suto *et al*. 2001).

2.2   Aetiology and prevalence

Ehrlichia and Anaplasma are alpha-proteobacteria within the family of *Anaplasmataceae*. They are obligate intracellular gram-negative pleomorphic cocci capable of causing disease in several species of domestic and wild animals, and humans (Ndip *et al*. 2005; Sumner *et al*. 2000). These organisms are found in membrane lined vacuoles within the cytoplasmic host cells, most often leukocytes. They grow within membrane bound cytoplasmic compartments, which do not fuse with lysosomes (Sumner *et al*. 2000). Many ehrlichia are tick borne, although there are some species which use other invertebrates as intermediate hosts, such as snails and helminths.

Three distinct groups of the genus Ehrlichia had been identified based on their genes and include the *Ehrlichia canis* genogroup (*E. canis, E. chaffeensis, E. muris, E. ewingii* and *Cowdria ruminatium*), *Ehrlichia phagocytophilia* genogroup (*E. equi, E. phagocytophilia* and *E. platys*) and *Ehrlichia sennetsu* genogroup (*E. sennetsu, E. risticii* and probably *Neorickettsia helminthoeca*) (Dumler *et al*. 1995). Molecular techniques have led to discoveries on these organisms culminating in a recent proposal for reclassification with changes in nomenclature. The *Ehrlichia canis* genotype retain the name, while the *Ehrlichia phagocytophilia* genotype changes from Ehrlichia to Anaplasma, and the members of *Ehrlichia sennetsu* become Neorickettsia (Cohn, 2003). These changes were proposed by Dumler *et al*. (2001) based on the degree of similarity of the 16S rRNA genes. The reclassification placed the organisms in the family *Anaplasmataceae* which include all species of the *alpha-Proteobacteria* originally in the genera *Ehrlichia*, *Anaplasma*, *Cowdria*, *Wolbachia* and *Neorickettsia. Ehrlichia ewingii* is a granulocytic species that has been isolated from dogs in the southern, western, and the Midwestern USA. *Ehrlichia ewingii* was first reported in 1971 by *Ewing* and others but was not considered a separate ehrlichial disease until 1985 (Stockham *et al*. 1985).A tropism for granulocytes differentiated *Ehrlichia ewingii* from the monocytotropic *Ehrlichia canis* but antigenic cross-reactivity was noted by western immunoblot analysis (Rikihisa *et al*. 1992).

Ehrlichia equi is closely related to the human granulocytic ehrlichiosis and infects dogs and horses in western USA, while *Ehrlichia phagocytophilia* infects dogs and ruminants in Europe. The two species have now been reclassified as one *Anaplasma phagocytophilum* (Dumler *et al*. 2001). *Anaplasma phagocytophilum* is the causative

agent of tick-borne fever in small ruminants and has been verified as the zoonotic agent of human granulocytic anaplasmosis (Stuen, 2007; Woldehiwet, 2006). *Ehrlichia platys* the cause of infectious thrombocytopenia infects only platelets resulting in minimal if any haemorrhagic tendencies in dogs. *Ehrlichia chaffeensis* a cause of human monocytic ehrlichiosis can experimentally infect dogs. Naturally occurring infection in dogs with *Ehrlichia chaffeensis* has close antigenic relationship with *Ehrlichia canis*. The true incidence of *Ehrlichia chaffeensis* is still unknown.

Experimentally, *Ehrlichia canis* infection has been demonstrated in other animals including monkeys, jackals, foxes and coyotes. It has been suggested that the wild dog *Lycaon pictus* could serve as a reservoir for ehrlichia species. A fatal case of ehrlichiosis has been reported in a silver- backed jackal *Canis mesomelas* after exposure to ticks. Two wild cats, a leopard and a Lynx were found to have pathological changes similar to Nairobi bleeding disease, seen in 11 dogs in Kenya (Murray, 1967).

Previously, infection with ehrlichia species has been considered to be host specific. *Ehrlichia canis* was thought to only infect dogs and *Ehrlichia chaffeensis* to infect humans and deer (Breitschwerdt *et al*. 1998) until an isolate genetically and antigenically similar to *Ehrlichia canis* was isolated from a man in Venezuela (Perez *et al*. 1996). Similarly, isolates genetically identical to *Ehrlichia risticii*, the cause of Potomac fever in horses, was obtained from dogs (Kakoma *et al*. 1994). There is also evidence indicating that members of the *Ehrlichia phagocytophilia* group cause disease manifestations in cats, dogs, horses and human beings (Greig *et al*. 1996). *Ehrlichia chaffeensis,* originally

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isolated and characterized as a cause of human disease has been isolated from dogs, and also found to cause severe disease manifestations in naturally infected dogs (Dawson *et al*. 1996; Breitschwerdt *et al*. 1998a). Feline granulocytic ehrlichiosis has been reported in a cat (Bjoersdorff *et al*. 1999).

Information on the prevalence of *Ehrlichia canis* among dogs detected by molecular techniques such as PCR is scanty. A study by Kordick *et al*. (1999) reported prevalence for *Ehrlichia canis* of 56% in a kennel in North Carolina as determined by PCR. Murphy *et al*. (1998) detected *Ehrlichia canis* DNA in 3% of dogs in Oklahoma. A study of Venezuelan dogs (Unver *et al*. 2001b) reported 31% positive by PCR specific to *Ehrlichia canis*. This high infection rate demonstrated that *Ehrlichia canis* is a common pathogen among the dog population in Venezuela and attention needs to be paid by both veterinarians and public health professionals in the area. Although ehrlichiosis is a common clinical entity, no current scientific reports are available indicating its prevalence in dogs in Kenya. The information on the prevalence of this condition would help elucidate the full extent of its clinical manifestations particularly where confirmatory diagnostic tools are unavailable.

Human ehrlichioses are tick borne illnesses of worldwide importance (Pritt *et al*. 2011; Ismail *et al*. 2010; Ravyn *et al*. 1999; Perez *et al*. 1996). In recent years, three Ehrlichia species have been newly recognized as human pathogens transmitted by ticks in the United States (Sumner *et al*. 2000). The most recently reported agent *Ehrlichia ewingii* is the etiologic agent of canine granulocytic ehrlichiosis (CGE). Canine granulocytic

ehrlichiosis was reported by Ewingii and others in 1971, but was not considered a separate ehrlichial disease until 1985 (Stockham *et al*. 1985). A tropism for granulocytes initially differentiated *Ehrlichia ewingii* from *Ehrlichia canis*, the etiologic agent of canine monocytic ehrlichiosis. It was recognized as a separate species in 1992 after molecular evaluation of the 16S rRNA gene sequence (Anderson *et al*. 1992). A number of reports characterizing the role of *Ehrlichia ewingii* in canine granulocytic ehrlichiosis (CGE) have been published (Breitschwerdt *et al*., 1998a; Goldman *et al*. 1998; Kordick *et al*. 1999; Murphy *et al*. 1998). *Ehrlichia ewingii* has been documented to infect humans (Buller *et al*. 1999).It is therefore necessary to perform species specific PCR in dogs to determine which Ehrlichia species are the causative agents of ehrlichiosis and to elucidate the potential of infection in humans in this region. The information would be of important clinical and epidemiological value and help improve the existing knowledge on tropical medicine.

2.3   Transmission

The ehrlichia organism is transstadially transmitted by the nymph and adult stages of the brown dog tick, *Rhipicephalus sanguineus* (Groves *et al*. 1975), which is endemic world-wide, and the adult stage of the American dog tick, *Dermacentor variabilis* (Johnson *et al*. 1998). The Lone Star tick, *Amblyomma americanum*, has been shown to experimentally transmit both *Ehrlichia chaffeensis* (Ewing *et al*. 1995) and *Ehrlichia ewingii* (Anziani *et al*. 1990). *Ehrlichia chaffeensis* has also been detected in the ticks *Dermacentor variabilis* and *Ixodes pacificus* (Kramer *et al*. 1999).This ehrlichia agent has also been identified in *Ixodes persulcatus* ticks (Kim *et al*. 2003).

Vectors for *Anaplasma phagocytophilum* include ticks of the *Ixodes persulcatus* complex (Telford *et al*. 1996; Baumgarten *et al*. 1999).*Anaplasma phagocytophilum* has also been identified in *Haemaphysalis longicornis* ticks (Kim *et al*. 2003). In North America, the black-legged tick, *Ixodes scapularis* is the principal vector for *Anaplasma platys*. Others are *Ixodes pacificus* and *Dermacentor spp*.

Rare examples of non-tick transmission of human granulocytic anaplasmosis exist in the literature and include direct exposure to deer blood (Bakken *et al*. 1996), transfusion (Leiby *et al*. 2004) and transplacental transmission (Horowitz *et al*. 1998). Under some circumstances, other rickettsial infections have been shown to be transmissible via aerosol, direct contact with mucous membranes or conjunctivae, or mechanical fomite transmission (Zhang *et al*. 2008; Diez *et al*. 1988; Hawkins *et al*. 1982; Kenyon *et al*. 1979; Oster *et al*. 1977).

2.4   Clinical Features of Ehrlichiosis

Experimental inoculations have demonstrated an incubation period of 8-20 days in which the bacteria spread throughout the body in the mononuclear-phagocyte system (Neer and Harrus, 2006). Ehrlichial infection may result in a wide variety of clinical signs of which depression, lethargy, weight loss, anorexia, pyrexia, lymphoadenopathy, splenomegaly and a tendency to haemorrhage are the most common especially for *Ehrlichia canis*. Pale mucous membranes, hepatomegaly and increased hair loss have being reported. Diarrhoea and vomiting have also been reported. Often, the only clinical signs of ehrlichiosis are pyrexia, apathy, weight loss and diarrhoea (Clark *et al*. 1996). Vomiting,

epistaxis, lymphadenopathy and anterior uveitis have also been documented in *Ehrlichia chaffeensis* infection. Other clinical signs reported are halitosis, serous to mucopurulent nasal and ocular discharges, gastritis, brown deposition on teeth, rapid and thready pulse, polyuria and pain over the bladder area on palpation of the abdomen. Panting has also been observed in a high percentage of dogs (Castro *et al*. 2004).

Photophobia and retinal vascular engorgement have been noted during the initial febrile period. This is followed by regression and simultaneous development of perivascular lesions in both tapetal and non- tapetal zones. Gould *et al*. (2000) have reported acute blindness of sudden onset in a Labrador retriever. This was associated with bilateral uveitis, intraocular, haemorrhage and retinal detachment. Retinal petechial haemorrhages have also been reported in *Anaplasma phagocytophilum* infection (Bexfield *et al*. 2005).

Other signs may include convulsions, hyperaesthesia, hysteria, paralysis, muscular weakness and partial paraplegia. In a retrospective study of *E. ewingii* infection, Goodman *et al*. (2003) observed ataxia, paresis, proprioceptive deficits, anisocoria, intention tremor, and head tilt. Lameness, polyarthritis, joint pain and swelling caused by effusion have also been reported (Bexfield *et al*. 2005; Goodman *et al*. 2003; Goldman *et al*. 1998).

In a study in dogs infected with *Anaplasma phagocytophilum* Poitout *et al.* (2005) reported fever, lethargy, and anorexia as the most common clinical signs. Bexfield *et al*. (2005) observed pallor of mucous membranes, petechiae, generalized lymphadenopathy,

effusions in multiple joints and mild oedema in a 10 year old dog with *Anaplasma phagocytophilum* infection.

Following the manifestations of the acute clinical phase of infection, the subclinical phase of persistent ehrlichial infection and mild thrombocytopenia may occur and may last for 40 to 120 days or years. The chronic phase of *Ehrlichia canis* infection is characterized by haemorrhages, epistaxis and oedema in addition to the clinical signs and laboratory findings of the acute phase, which are often complicated by super infection with other organisms (Unver *et al*. 2001b).

2.5   Haematological changes in Ehrlichiosis

The principal haematological abnormalities include thrombocytopenia, mild anaemia and mild leucopenia during the acute form of the disease, mild thrombocytopenia in the sub clinical form, and pancytopenia in the severe chronic form (Harrus *et al*. 1999). A normocytic, normochromic anaemia also occurs (Goldman *et al*. 1998; Kuhen and Gaunt, 1985). The anaemia associated with all ehrlichial infections is classically non-regenerative and occurs in the chronic phase of the disease, due to suppressive effects of the parasite on the bone marrow (Neer *et al*. 2002).

Thrombocytopenia is the most prominent and consistent haematological change occurring in humans and animals infected by a wide range of *Ehrlichia* species (Goldman *et al*. 1998; Clark *et al*. 1996; Harrus *et al*. 1997b; Egenvall *et al*. 1997; Neer *et al*. 2002). Thrombocytopenia and lymphopenia have also been reported in *Anaplasma phagocytophilum* infection in dogs (Poitout *et al*. 2005). The thrombocytopenia has an

immunological component and *Ehrlichia canis* is postulated to trigger autoimmune mechanisms in the dog (Waner *et al*. 2000a) as the presence of circulating serum antibodies have been demonstrated (Waner *et al*. 1995). However, other mechanisms may also be involved in the development of thrombocytopenia in canine monocytic ehrlichiosis. Proposed mechanisms include increased platelet consumption or separation (Smith *et al*. 1974; Ristic and Holland 1993); splenic pooling in enlarged spleens, and increased platelets destruction by the spleen (Smith *et al*. 1974) and suppression of platelet production in the bone marrow, mainly in the chronic phase (Woody and Hoskins, 1991). Radio labelled platelet survival time decreases from 9 days to 4 days, 2-4 days after artificial infection with *Ehrlichia canis* (Smith *et al*. 1974). Whole body scans on a dog before and 7 days after infection with *Ehrlichia canis* have shown that labelled platelets are destroyed primarily in the spleen, similar to what occurs in immunologically mediated thrombocytopenia purpura in man (Smith *et al*. 1974).

In a retrospective study, Harrus *et al*. (1997a) concluded that severe anaemia, severe leucopenia, pancytopenia, a tendency to bleed (especially epistaxis) and being a German shepherd dog were important indicators of poor survival in cases of monocytic ehrlichiosis in dogs.

2.6   Biochemical changes in Ehrlichiosis

Significant alterations occur in the serum protein profile of dogs naturally and artificially infected with *Ehrlichia canis* (Harrus *et al*. 1996). Hypergammaglobulinaemia is one of the main biochemical abnormalities of *Ehrlichia canis* infection. It is usually polyclonal;

monoclonal gammopathy is rare (Harrus *et al*. 1996). Hypoalbuminemia and elevated alkaline phosphatase, alanine amininotransferase (Frank and Breitschwerdt, 1999; Waddle and Littman, 1988), serum lactate dehydrogenase, blood urea concentrations, creatinine concentrations have been reported (Harrus *et al*. 1997a). Proteinuria may occur independently or concurrently with glomerulonephritis (Frank and Breitschwerdt, 1999; Waddle and Littman, 1988; Codner and Maslin, 1992).

Elevated activity of alkaline phosphatase in serum has been reported as a common laboratory finding in *Anaplasma phagocytophilum* infection in dogs (Poitout *et al*., 2005). Bexfield *et al*. (2005) reported mild increase in alkaline phosphatase, total bilirubin and creatinine in a 10 year crossbreed dog.

2.7   Immunological Features of Ehrlichiosis

Intracellular bacteria are believed to have established the ability to replicate in the host cell in order to evade the immune system. This works as a protective mechanism by evading antibodies which cannot enter the host cell (Perez*etal*.1996). Although the ehrlichiae are thought to be highly host specific, the immunologic responses among the monocytophilic ehrlichiae appear to be similar (Brouqui and Dumler, 1997).

The primary but not exclusive mechanism of immunity to obligate intracellular bacteria is the cell-mediated immune response and especially the delayed-hypersensitivity response (Jerrells, 1997). Killing of intracellular bacteria by monocytes involves oxygen dependent and independent mechanisms. Oxidative mechanisms appear to play a minor role in

killing of intracellular Ehrlichiae (Brouqui and Dumler, 1997). The survival and multiplication of *Ehrlichia sennetsu* and *Ehrlichia risticii* in infected cells has been demonstrated to rely on their ability to inhibit phagosome-fusion (Park and Rikihisa, 1991; Wells and Rikihisa, 1988).

The role of humoral antibody response is unclear, and in some cases may have a detrimental effect on the pathogenesis of the disease (Harrus *et al*. 1999). Passive transfer of immune sera that contain high levels of antibodies have failed to protect, when given either before or after infection (Jerrells, 1997).

Rickettsial agents establish a long-term carrier state, avoiding the host immune responses (Reddy *et al*. 1998) but the exact mechanism of persistence has not been clarified. It has been proposed that the presence of multiple genes containing constant and variable regions in the ehrlichiae may provide opportunities of recombination leading to variation in immunologic surface epitopes (Reddy *et al*. 1998). By varying the sequence of hypervariable immunologic epitopes, it is possible that Ehrlichiae could persist by evading the host immune responses (McBride *et al*. 1996).

Infection with *Ehrlichia canis* results in the development of specific antibodies. In experimental infections with blood from *Ehrlichia canis* infected dogs, IgG and IgA antibodies appear about 4-7 days after infection and 15 days later IgG antibodies are detected post infection (Waner *et al*., 1996). Variations in the first appearance of *Ehrlichia canis* specific antibodies have been reported in the literature. The initial

appearance of IgG antibodies appears to be related to the dose of the infective organisms to which the dog is exposed (Rikihisa *et al*. 1992).

Coombs positive immune-mediated haemolytic anaemia or evidence of autoagglutination has been associated with canine monocytic ehrlichiosis (Frank and Breitschwerdt, 1999; Breitschwerdt, 2000). In addition, anti-erythrocyte antibodies and positive in-saline agglutination have been detected in sera of several dogs infected with granulocytic *Ehrlichia* strain in the USA (Goldman *et al*. 1998).

2.8   Molecular Biology of Ehrlichia Species

Phylogenetic analysis based on the housekeeping genes such as 16S rRNA gene DNA sequences suggests that Ehrlichia species were derived from a common ancestor. The 16S rRNA gene DNA sequences are highly conserved among strains of each Ehrlichia species (Yu *et al.* 2007). Ehrlichia species are classified on the basis of their 16S rRNA gene sequence (Hildebrandt *et al*., 2002).Molecular and antigenic analyses, particularly the comparison of 16S rRNA gene sequences, enabled the segregation of Ehrlichia species into three monophyletic clades commonly referred to as genogroups which include the *Ehrlichia canis* genogroup (*E. canis, E. chaffeensis, E. muris, E. ewingii* and *Cowdria ruminatium*), *Ehrlichia phagocytophila* genogroup (*E. equi, E. phagocytophila* and *E. platys*) and *Ehrlichia sennetsu* genogroup (*E. sennetsu, E. risticii* and probably *Neorickettsia helminthoeca*) (Sumner *et al*. 2000; Dumler *et al*. 1995).

Based on the degree of similarity of the 16S rRNA genes of the species, Dumler *et al.* (2001) proposed a new classification of these pathogens. These are *Anaplasma phagocytophilum* which joined together three previously described species *Ehrlichia equi, Ehrlichia phagocytophilum*, and human granulocytic agent (HGE agent). *Ehrlichia sennetsu* is reclassified as *Neorickettsia sennetsu*, *Ehrlichia canis*, *Ehrlichia ewingii* and *Ehrlichia chaffeensis* retained their classification.

Ehrlichia ewingii is a granulocytic specific species that has been isolated from dogs in the southern, western and Midwestern USA. It was first reported in 1971 by Ewing and others but was not considered a separate ehrlichial disease until 1985 (Stockham *et al.* 1985). A tropism for granulocytes initially differentiated *Ehrlichia ewingii* from *Ehrlichia canis* the etiologic agent of canine monocytic ehrlichiosis. However, antigenic cross-reactivity between *Ehrlichia ewingii* and the monocytotropic *E. canis* by western immunoblot analysis was noted (Rikihisa *et al.* 1992). *Ehrlichia ewingii* was later recognized as a separate species, when the 16S rRNA gene sequence was shown to be different from the corresponding sequences of the closely related species, *Ehrlichia canis* and *Ehrlichia chaffeensis* (Anderson *et al.* 1992). Nucleotide sequences that match the *Ehrlichia ewingii* 16S rRNA gene have been amplified from blood samples of human beings (Buller *et al.* 1999) and this was the first documented case of human ehrlichiosis caused by *Ehrlichia ewingii*.

All Ehrlichia species have a p28 gene family, which consists of multiple copies of homologous genes encoding 28–30 kDa outer membrane proteins which have been used

to study the diversity of ehrlichia organisms. These p28 genes are highly diversified among strains of *Ehrlichia chaffeensis* and *Ehrlichia ruminantium*, but very conserved among *Ehrlichia canis* strains (Yu *et al.* 2007). The genetic restriction of *Ehrlichia canis* and divergence of *Ehrlichia chaffeensis* has been further confirmed by analysis of other surface protein genes suchasgp120/gp140andvlptgene (Yu *et al.* 2007). Other genes that have been used in the study of ehrlichia are the disulfide bond formation protein gene (dsb) (Labruna *et al.* 2007).

Previously, infection with ehrlichia species has been considered to be host specific. *Ehrlichia canis* was thought to only infect dogs and *Ehrlichia chaffeensis* to infect humans and deer (Breitschwerdt *et al.*, 1998a). However, an isolate genetically and antigenically similar to *Ehrlichia canis* was obtained from a man in Venezuela (Perez *et al.* 1996). Similarly, isolates genetically identical to *Ehrlichia risticii*, the cause of Potomac fever in horses, have been obtained from dogs (Kakoma *et al.* 1994).

2.9   Pathological Alterations

The gross lesions observed in canine ehrlichiosis are haemorrhages in the subcutaneous tissues and major organs, generalized lymphadenopathy with mesenteric nodes more commonly affected, and oedema of the limbs. The distribution and severity of haemorrhages varies although the heart, lungs and gastrointestinal and urogenital tracts are most affected (Castro *et al.* 2004; Bexfield *et al.* 2005).

Emaciation with little subcutaneous fat, pale mucous membranes, subcutaneous tissues and musculature has been reported. The spleen is enlarged and the liver may be pale and icteric or enlarged. Paleness of the kidney and liver, ascites, congestion of the lungs have also been reported (Castro *et al*. 2004). Congestion of the spleen has also been observed (Bexfield *et al*. 2005).

Microscopic examination showed a reactive hyperplasia in the spleen, a reactive hepatitis in the liver and microthrombi in capillaries of the renal glomeruli and lungs of a dog that had *Anaplasma phagocytophilum* (Bexfield *et al*. 2005). Scattered lobular lymphohistiocytic foci and diffuse lymphohistiocytic infiltration and Kupffer cell hyperplasia have been reported in livers from humans infected with human monocytic ehrlichiosis (HME) caused by *Ehrlichia chaffeensis* and cholestasis with bile duct epithelial injury was also noted (Sehdev and Dumler, 2003).

2.10   Diagnostic Methods in Ehrlichiosis

Identification of morulae in blood smears is diagnostic. Although the search for morulae in circulating monocytes is important, it may be unrewarding (Woody and Hoskins, 1991); this is due to the frequently low parasitaemia. Blood smears made from the first drop that emerged from the tip of the ear, were demonstrated to be most satisfactory for the demonstration of *Ehrlichia canis*. Morulae of *Ehrlichia ewingii* are found in neutrophils and eosinophils upon examination of Giemsa stained blood smears during acute rickettsemia (Cohn 2003; Goodman *et al*. 2003).

Nyindo *et al*. (1971) developed an *in vitro* cell culture technique and were able to study elementary, initial bodies and morulae of *Ehrlichia canis* in mononuclear cells. This technique has been used as a confirmatory diagnosis for ehrlichiosis. An adaptation of this technique was described in detail by Kaminjolo *et al.* (1976) in which they used foetal bovine serum in place of canine serum. *Ehrlichia ewingii* has, however, not yet been isolated in cell culture. Generally, culture of intracellular organisms is difficult and expensive and is used primarily in a research setting than for clinical disease diagnosis.

An indirect florescent antibody (IFA) test was developed by Ristic *et al*. (1972), which is specific and reliable for diagnosis of *Ehrlichia canis* infection. The test has played a crucial role in research and diagnosis of the disease and though other diagnostic methods have been developed, the IFA test remains in common use (La Scola and Roult, 1999). Serologic cross-reactivity between ehrlichial species may pose a serious problem in the interpretation of IFA results (Neer, 1998). The use of IFA technique does not facilitate the differentiation of the infecting Ehrlichia species, particularly among organisms of the same genogroup (Warner *et al*. 2001). Moreover, the IFA test is generally available in selected laboratories and requires expensive equipment and trained personnel.

The use of an enzyme- linked immunosorbent assay (ELISA) test for the early diagnosis of canine monocytic ehrlichiosis by the detection of plasma ehrlichial antigen has been tested in experimentally infected dogs and found to be unreliable (Warner *et al*. 1996). On the other hand, Harrus *et al*. (2001) developed an ELISA method for the detection of anti-ehrlichia canis antibodies. The total IgG ELISA results obtained in their study were sensitive and specific for *Ehrlichia canis* and with a significant correlation with the IFA

test. Early detection of IgG antibodies against *Ehrlichia canis* by the ELISA test has been reported, where the results correlated well with the appearance of fever and clinical signs of ehrlichiosis (Warner *et al.* 2000b).

A highly specific and sensitive Polymerase Chain Reaction (PCR) assay for *Ehrlichia canis* based on the 16S sequence of the Lousiana isolate of *E. canis* has been developed (McBride *et al*. 1996).Several studies have shown PCR to be an effective and extremely sensitive method for the detection of *Ehrlichia* and *Anaplasma* species in dog blood and tissues (Dawson *et al.* 1996; Engvall *et al.* 1996; McBride *et al*. 1996; Iqbal and Rikihisa 1994a; Iqbal *et al*. 1994). The test has been widely used in the laboratory diagnosis of canine monocytic ehrlichiosis (CME), especially during the acute phase of the disease before antibodies are detectable (Wen *et al*. 1997). Engvall *et al*. (1996) found PCR to be the most reliable method and useful in the clinical laboratory for specific and early diagnosis of granulocytic ehrlichiosis in animals. Detection and diagnosis of infection of monocytic ehrlichiosis by PCR in serum has been reported (Mylonakis *et al*. 2009). Alexandre *et al*. (2009) reported detection of *Ehrlichia canis* by nested PCR in dogs that were seronegative by indirect IFA. This reinforces the value of molecular techniques in the early diagnosis of CME (Alexandre *et al*. 2009).

No scientific reports are available describing molecular studies of ehrlichial infections in dogs in Kenya. Such studies would be useful in further understanding the etiology, prevalence and clinico-pathological manifestations of this disease in dogs in Kenya. This

would facilitate accurate diagnosis, rational medical management and institution of sound advice to clients on preventive measures.

This study was therefore undertaken to establish by molecular techniques the species of ehrlichia organisms present in Kenya. The information will improve knowledge and practice relating to the clinical presentation, management and public health importance of ehrlichial infections in the region.

CHAPTER 3

3.0. A retrospective study of clinical presentation and treatment of Canine Ehrlichiosis at the Small Animal Clinic, University of Nairobi

3.1. Introduction

Ehrlichioses are tick borne diseases caused by obligate intracellular α-proteobacteria belonging to the genera *Ehrlichia* and *Anaplasma* respectively. *Ehrlichia canis* was first recognized as a distinct clinical entity in Algeria in 1935. Infection occurs worldwide in dogs and other canids with a higher frequency in tropical and sub-tropical regions (Alexandre *et al*. 2009; Suto *et al*. 2001). The pathogens are classified as *Anaplasma phagocytophilum, Neorickettsia sennetsu*, *Ehrlichia canis*, *Ehrlichia ewingii* and *Ehrlichia chaffeensis* (Dumler *et al.* 2001).

The classic ehrlichiosis is an acute to chronic disease caused by *Ehrlichia canis.* Acute canine monocytic ehrlichiosis may be manifested in dogs by symptoms such as fever, depression, dyspnoea, anorexia and slight weight loss. This is followed by a subclinical phase of persistent ehrlichial infection and mild thrombocytopenia, lasting 40 to 120 days or years. The chronic phase is characterized by haemorrhages, epistaxis and oedema, in addition to the clinical signs and laboratory findings of the acute phase, often complicated by superinfection with other organisms (Unver *et. al*. 2001b). Often the only clinical signs observed in ehrlichiosis are pyrexia, apathy, weight loss and diarrhoea (Clark *et al*. 1996). Other clinical signs reported are panting (Castro *et al*. 2004), blindness (Gould *et al*. 2000), retinal haemorrhage (Bexfield *et al*. 2005) and lameness (Bexfield *et al*. 2005; Goodman *et al*. 2003; Goldman *et al.* 1998).

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The disease was first reported in dogs in Nairobi, Kenya by Danks (1937) and Murray (1968). However, at this stage, the etiological cause was unknown. *Ehrlichia canis* was later confirmed in East Africa using cell culture isolation and indirect florescent antibody tests (Kaminjolo *et al*. 1976) and serology in free ranging jackals in Kenya (Alexander *et al*. 1994). Price (1980) has described clinical and haematological features of natural and experimental canine ehrlichiosis.

Although ehrlichiosis is a common clinical entity, current information in published scientific literature on the clinical presentation of the disease in Kenya is scanty. Such information is important in understanding the clinical presentation of canine ehrlichiosis and aid practitioners in making tentative diagnosis of the infection. This study was undertaken to describe the presenting clinical features and evaluate the treatment outcomes in dogs diagnosed with ehrlichiosis at the Small Animal Clinic during the period between 1993 and 2006.

3.2. Materials and methods

3.2.1. Data collection

Data was obtained from records of 514 dogs diagnosed with Ehrlichiosis at Small Animal Clinic (SAC), Faculty of Veterinary Medicine, University of Nairobi, Kenya from 1993 to 2006. The dogs were from Nairobi and its environs. Data were collected from the details available in the record cards for each specific case.

Records of all cases were retrieved from the files through the case lists in the annual catalogues from the archives. The medical files of the dogs with a diagnosis of

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ehrlichiosis were retrieved. From these, those with confirmed diagnosis of ehrlichia by either blood smear or *in vitro* cell culture (Nyindo's test) were identified. Records were examined to obtain information on breed, age, sex, clinical history, clinical signs, treatment administered and outcome. The data collected was for the first visit when the animals were diagnosed with ehrlichiosis and the second visit 14 days later when they were returned for final treatment or check-up.

The data recorded for final computation and analysis included breed, sex, clinical signs, treatment and outcome. The data was recorded in data collection sheet (Appendix 1).The findings were coded as "1" (meaning the clinical observation was present) and "0" (meaning the clinical observation was not present).

3.2.2. Statistical Analysis

The data were stored in Microsoft Office Excel 2007 (Microsoft Corporation, 2007) and exported to SPSS and Genstat for Windows Edition 2 (VSN International) software for analysis. Descriptive statistics were done for clinical signs observed and treatments. Parameters between the first and second visits to the clinic were compared using Independent t-test to determine statistical significance of any differences at a confidence interval of 95% ($p \leq 0.05$).

3.3 Results

3.3.1. Signalment

There were 54.4% males and 45.4% females among the dogs with clinical ehrlichiosis in the period under review. The German shepherd dog was the most common breed with 42.6% (204/479) of all the ehrlichia infected dogs seen at the Small Animal Clinic. Cross-breeds were 28.6 % (137/479), German shepherd crosses 4.6 % (22/479), Ridgebacks 4 % (19/479) and Rottweilers 4 % (19/479). A summary of the breed of dogs seen at the Small Animal Clinic with ehrlichiosis is presented in Table 1. Information on the age was not consistently recorded in the patients' records and was therefore not analysed in this study.

Table 1. Percentage of dog breeds diagnosed with clinical ehrlichiosis in the Small Animal Clinic, Faculty of Veterinary Medicine, University of Nairobi, during the period 1993-2006.

Dog breed	Percentage
German Shepherd	42.6 (204/479)
Labrador	1.9 (9/479)
Rottweiler	4 (19/479)
Ridgeback	4 (19/479)
Doberman	2.5 (12/479)
Spitz	1 (5/479)
Cross breeds	28.6 (137/479)
German Shepherd crosses	4.6 (22/479)
Local	0.4 (2/479)
Other breeds (Retrievers, spaniels, terriers, etc)	10.4 (50/479)

3.3.2 Clinical signs in confirmed cases of ehrlichiosis

The most common clinical signs in dogs diagnosed with ehrlichiosis were lymphadenomegaly (59.3%), congestion of mucous membranes (48.7%), inappetence (43.7%), a pounding heart (28.4%), pale mucous membranes (21.3%), harsh lung sounds (20.4) and vomiting (19.8%). The clinical signs upon presentation to the clinic are summarized in Table 2.Concurrent infections were identified in 36.4% (181/497) of the ehrlichia infected dogs. Six point six percent (33/497) of these infections were by Babesia and 6.4% (32/497) were by intestinal helminths.

Table 2. Clinical signs noted in dogs diagnosed with clinical ehrlichiosis in the Small Animal Clinic, Faculty of Veterinary Medicine, University of Nairobi, during the period 1993-2006.

Clinical sign	Percentage
Lethargy	21.2 (70/331)
Weakness	8.3 (35/421)
Inappetence	43.7 (201/460)
Pale mucous membranes	21.3 (97/455)
Congestion of mucous membrane	48.7 (221/454)
Ocular discharge	8 (34/426)
Harsh lungs	20.4 (34/167)
Panting	8.8 (33/331)
Vomiting	19.8 (34/172)
Splenomegaly	14 (64/457)
Lymphadenomegaly	59.3 (267/450)
Tender abdomen	14.9 (68/457)
Diarrhoea	13.3 (62/465)
Haemorrhage	11.7 (60/514)
Hind limb weakness	3.3 (15/451)
Pounding heart	28.4 (146/514)
Flea allergy dermatitis	2 (9/458)
Dermatitis	3.3 (17/512)

3.3.3 Treatment and outcome

Eighty two point six percent (419/507) of the dogs were treated with Imidocarb dipropionate at a dose of 5mg/kg body weight intramuscular injection repeated after 2 weeks. Two point eight percent (14/507) were treated with oral doxycycline at a dose of 10mg/kg. The other treatments were oral tetracycline at 66mg/kg divided dose 0.4% (2/507) or a combination of Imidocarb dipropionate with either tetracycline or doxycycline 14% (71/507).

Fifty six point five percent (275/487) of the treated dogs were presented back after 2 weeks for follow up treatment and assessment. Of the treated animals 49.1% (239/487) improved with resolution of the clinical observations that had been present in the dogs, and 7.4% (36/487) showed no improvement at all. Forty three point five percent (212/487) were not presented for follow up but some were latter seen at the clinic for other reasons as noted from the dogs' medical record.

3.4 Discussion

An important observation in this study was the fact that clinical data on the various parameters under consideration were not consistently recorded by clinicians. This explains the variations in the denominators in the tables. However, comparison of the proportion of parameters expressed as percentages is an appropriate mode of presenting data generated from retrospective studies.

In this study, the larger proportion of German shepherd dogs that was observed with the infection could be due to higher susceptibility by this breed to ehrlichiosis. Previous studies have reported this breed to be more susceptible to ehrlichia (Harrus *et al*. 1997a; Rikihisa 1991; Nyindo *et al*. 1980.Harrus *et al*. (1997a) also noted over-representation of the German shepherd dog with a concurrent under-representation of cross breeds in dogs suffering from canine monocytic ehrlichiosis. This finding therefore confirms the observation that the German shepherd dog is very susceptible to infection by ehrlichia. However, it could also be due to their higher proportion among the dogs attended at the clinic in the period covered by the study. It is also important to note that the German shepherd dog is a very popular breed in Kenya and this could also be the reason for their larger number among the ehrlichia infected dogs.

The observation in the study that males were over represented at 54.4% among the dogs diagnosed with ehrlichia was comparable to the report by Ndip *et al*. (2005) in a study in Cameroon which noted that the majority of dogs affected by ehrlichia were males at 63%. This finding is, however, in contrast with the observation by Harrus *et al*. (1997a) that noted no sex predilection in a retrospective study of canine monocytic ehrlichiosis. This over representation by males could be due to bias by most dog owners for males as compared to females based on reproductive behaviour. In Kenya, a notable bias for male dogs by security agencies may be due to reproductive behaviour. It is probable that this may have influenced the gender of dogs presented for treatment at practice facilities.

Naturally occurring canine monocytic ehrlichiosis may be manifested by a wide variety of clinical signs (Harrus *et al*. 1997b) as the disease affects different systems in infected animals. The clinical signs that have been reported for ehrlichia infection include lethargy, anorexia, fever, panting, lymphadenomegaly, splenomegaly, weight loss, pale mucous membranes and bleeding (Shipov *et al*. 2008; Mylonakis *et al*. 2008; Harrus *et al*. 1997a; Neer, 1995). The observed clinical signs in this study were lethargy, anorexia, fever, panting, lymphadenomegaly, pale mucous membranes, congestion of mucous membranes, pounding heart, harsh lungs, vomiting, tender abdomen, splenomegaly, ocular discharge, hind limb weakness, dermatitis, diarrhoea and haemorrhage. These signs are nonspecific and may generally not lead one to make a diagnosis of any specific infection in a dog. An important observation of this study is that lymphadenomegaly is the most common clinical sign present in dogs suffering from ehrlichia. This might be due to the multiplication of the parasite in the animal's organs and hence resulting in inflammatory response. Moreover, microscopic examination has demonstrated a reactive hyperplasia in the spleen and a reactive hepatitis in the liver of a dog infected by *Anaplasma phagocytophilum* (Bexfield *et al*. 2005). A similar mechanism may also explain the enlargement of the lymph nodes as observed in this study. The observation that lymphadenomegaly is a common finding in ehrlichial infections noted in this study, is in agreement with the finding by Harrus *et al*. (1997c) that lymphadenomegaly was one of the principal findings in dogs infected by *Anaplasma platys*.

Congestion of mucous membranes (48.7%) and inappetence (43%) observed in the dogs diagnosed with ehrlichiosis in this study also confirm these as important clinical

indicators of ehrlichial infection. Congestion of the mucous membranes occurred more frequently than pallor of the mucous membrane in dogs diagnosed with ehrlichiosis. This report contributes to filling information gap in scientific literature on congestion as a clinical feature in canine ehrlichiosis and anaplasmosis. Reported signs in literature are usually haemorrhages such as petechiae, ecchymoses and epistaxis (Harrus *et al*. 1997a, 1997b; Bexfield *et al*. 2005).

Granick *et al*. (2009) observed that lethargy is one of the clinical signs that may lead one to suspect a dog to be suffering from infection with *Anaplasma phagocytophilum*. In the present study, lethargy was noted in 21.2% of the cases, the low percentage may be due to the fact that the data did not capture the different genera or species of the infecting Anaplasmataceae. A study by Mazepa *et al*. (2010) reported lethargy, inappetence and fever to be the most common clinical signs in dogs with *Anaplasma phagocytophilum* infection.

An important epidemiological and diagnostic feature was the observation that some animals had concomitant infection, Babesia being the most frequent in association with Ehrlichiosis. Association of Ehrlichia with other hematozoa can be attributed to the presence of the common tick vector *Rhipicephalus sanguineus* which is also the transmitter of Babesia organisms. Studies on tick biology indicate that a small percentage of ticks are responsible for harbouring multiple pathogens and successfully transmitting all the pathogens to the host (Kaur *et al*. 2011).

Appropriate treatment for infection by Ehrlichia and Anaplasma results in complete recovery (Shipov *et al*. 2008). This study has established that the treatment preferred in the Small Animal Clinic, Faculty of Veterinary Medicine, University of Nairobi was intramuscular injection of Imidocarb dipropionate administered at a dosage of 5mg/kg body weight (82.6%) with only a negligible percentage using oral Doxycycline at 10mg/kg body weight (2.8%). The preference of Imidocarb dipropionate, an injectable drug, could be due to the ease of administration and maybe suspected lack of compliance by owners if doxycycline, an orally administered drug, is used. The main challenge revealed by this study was lack of follow up with the administered treatment. Of the treated dogs 49.1% were reported to have recovered but treatment could not be followed up in 43.5%. Follow up treatment is necessary when Imidocarb dipropionate is used in order to achieve complete elimination of the parasite. With the assumption that most of the animals that could be followed up may have shown clinical improvement and the owners saw no need of presenting them for the required second treatment with Imidocarb dipropionate, the recovery rate with this treatment is very high considering that only a very low percentage (7.3%) of the animals that were bought back for the second treatment in the study showed no improvement at all. However, this lack of follow up treatment does not augur well with the proper management of the infection as it is reported that with improper treatment or lack thereof, the disease may progress to the subclinical phase which may last for years (Shipov *et al*. 2008). In this subclinical phase the dogs appear healthy but may have mild thrombocytopenia (Waner *et al*. 1997; Harrus *et al*. 1998). Dogs in this phase may remain carriers for life, may spontaneously recover,

or may progress to the chronic severe form of the disease (Harrus *et al*. 1997a, 1997b; Waner *et al*. 1997).

In conclusion, it is noted that congestion of mucous membranes and inappetence are important clinical signs that can lead one to suspect a dog to be suffering from ehrlichiosis. It is also noted that to ensure dog owner's compliance with the requisite second visit for Imidocarb dipropionate treatment, client education may need to be done to ensure clearance of the ehrlichia from the treated dog. The Veterinary practitioners need to be encouraged to adopt the use of other drugs in the clinical management of ehrlichial infection especially doxycycline which is the drug of choice in other regions of the world.

CHAPTER 4

4.0 Prospective study of Ehrlichiosis and Anaplasmosis in dogs in Nairobi, Kenya

4.1 Introduction

Canine Ehrlichiosis and Anaplasmosis are globally recognized tick-borne diseases that occur mainly in the tropical and sub-tropical regions (Alexandre *et al*. 2009; Suto *et al*. 2001; Ravyn *et al*. 1999). They are caused by gram negative, obligate intracellular bacteria belonging to the family *Anaplasmataceae* capable of causing disease in animals and humans (Stuen, 2007; Ndip *et al*. 2005; Sumner *et al*. 2000; Greig *et al*. 1996). Within monocytes and granulocytes, the bacteria reside in inclusion bodies, where they are referred to as morulae (Woldehiwet *et al*. 2006; Sumner *et al*. 2000; Nyindo *et al*. 1971).

Ehrlichial infection may result in a wide variety of clinical signs; these include depression, lethargy, weight loss, anorexia, pyrexia, lymphoadenopathy, splenomegaly, haemorrhage, pale mucous membrane, hepatomegaly, increased hair loss, panting, diarrhoea and vomiting (Poitout *et al,* 2005; Castro *et al*. 2004; Clark *et al*. 1996). Gould *et al*. (2000) reported acute blindness of sudden onset in a Labrador retriever which was associated with bilateral uveitis, intraocular haemorrhage and retinal detachment. Other signs associated with *Anaplasma phagocytophilum* include retinal petechial haemorrhages and mild oedema (Bexfield *et al*. 2005). In a retrospective study of *Ehrlichia ewingii* infection, Goodman *et al*. (2003) observed ataxia, paresis, proprioceptive deficits, anisocoria, intention tremor, and head tilt. Lameness,

polyarthritis, joint pain and swelling caused by effusion have also been reported (Goodman *et al*. 2003; Bexfield *et al*. 2005).

The principal haematological abnormalities associated with *Ehrlichia* and *Anaplasma* infection include thrombocytopenia, mild anaemia and mild leucopenia during the acute form of the disease, mild thrombocytopenia in the sub clinical form, and pancytopenia in the severe chronic form (Harrus *et al*. 1999). A non-regenerative and normocytic, normochromic anaemia also occurs (Neer *et al*. 2002; Kuhen and Gaunt, 1985).Thrombocytopenia is the most prominent and consistent haematological change occurring in humans and animals infected by *Ehrlichia* species (Clark *et al*. 1996; Harrus *et al*. 1997b; Egenvall *et al*. 1997; Neer *et al*. 2002). Thrombocytopenia and lymphopenia have been reported in *Anaplasma phagocytophilum* infection in dogs (Poitout *et al*. 2005).

In a retrospective study, Harrus *et al*. (1997a) concluded that severe anaemia, severe leucopenia, pancytopenia, a tendency to bleed (especially epistaxis) and being a German shepherd dog were important indicators of poor survival in cases of monocytic ehrlichiosis in dogs.

Ehrlichia canis species infects monocytes and is the cause of canine monocytic ehrlichiosis. *Anaplasma platys* species infects platelets and causes canine cyclic thrombocytopenia. On other hand *Anaplasma phagocytophilum* infects granulocytes and causes granulocytic anaplasmosis formally known as granulocytic ehrlichiosis.

Anaplasma phagocytophilum, the causative agent of tick-borne fever in small ruminants and canine granulocytic anaplasmosis has been verified as the zoonotic agent of human granulocytic anaplasmosis (Stuen, 2007; Woldehiwet, 2006). *Anaplasma platys* platelet tropism is unique among ehrlichial-related organisms, even though all infections by these organisms may result in thrombocytopenia (Cohn, 2003). The dog is the primary reservoir for *Anaplasma platys* and has not been shown to infect humans (Gaunt *et al*. 2010).

Although ehrlichiosis is a common clinical entity (Alexander *et al*. 1994; Price 1980; Kaminjolo *et al*. 1976; Murray 1968; Danks 1937) scientific reports indicating the clinical, haematological and biochemical features of natural infection in Kenya are scant. This study was therefore undertaken to evaluate the clinical features, describe the haematological and blood chemistry profiles of ehrlichiosis and anaplasmosis in dogs presented at the Small Animal Clinic. The information would elucidate the full extent of its clinical features, particularly where confirmatory diagnostic tools are not readily available.

4.2 Materials and Methods

4.2.1 Study area and animal identification

In the period 2006 to 2009, 7012 dogs were seen at the Small Animal Clinic of which 60.9% (4268/7012) were presented for medical or surgical attention. The dogs were from Nairobi and the surrounding area. From the 4268 dogs presented for treatment 192 met the criteria for inclusion in this study. The criteria for inclusion in the study were clinical

signs suggestive of ehrlichia infection and presence of morulae in white blood cells on blood smears. Dogs with the clinical signs but with no morulae were excluded.

Thin blood smears from the micro-capillary circulation (ear tip) and also from EDTA-anticoagulated whole blood from each dog were prepared, air dried, fixed with methanol, stained with Giemsa as per the standard protocol (Coles, 1986). Microscopic examination of the blood smears was performed at 1000 times magnification under oil immersion. Leukocytes in the blood smears were examined for the presence of inclusion bodies also referred to as morulae, in monocytes and granulocytes.

4.2.2 Hematological profile

Two millilitres of blood was aseptically collected from the jugular vein by venepuncture into plastic tubes containing ethylenediaminetetraacetic acid (EDTA). Haematological assays were performed on the same day using a semi-automatic impedance cell counter (Compteur Analyseur d'Hematologie MS4, Melet Schoesing Laboratoires, 9 Chaussee Jules Cesar 95520 OSNY France). The following parameters were determined: packed cell volume, haemoglobin concentration, total erythrocyte count, mean corpuscular haemoglobin concentration, total and differential white blood cell count and total platelet count. The mean results were compared with normal reference values.

4.2.3 Blood chemistry

Five millilitres of blood was aseptically collected from the jugular vein by venepuncture into plain plastic tubes (with no anticoagulant).Serum separated by centrifugation two hours after the blood was drawn. Serum was analysed for gammaglobulins, albumins, alkaline phosphatase, alanine aminotransferase, blood urea, protein and creatinine. This was done using reagents from DiaSys Diagnostics GmbH Alte Strasse 9 65558 Holzheim Germany and a spectrophotometer (Visual 60V B0357 BioMerieux, sa 69280 Marcy I'Etoile, France). The mean results were compared with normal reference values.

4.2.4 Treatment

Animals diagnosed as ehrlichia positive on blood smears were treated with either Imidocarb dipropionate at a dose of 5 mg/kg body weight intramuscularly, doxycycline 10 mg/kg body weight orally or a combination of the two drugs. Information on response was recorded 14 days post treatment.

4.2.5Statistical Analyses

The results of the clinical examinations were coded as "1" (meaning the clinical observation was present) and "0" (meaning the clinical observation was not present). The data on breed, age, history, clinical signs, haematology, blood chemistry and treatment were stored in Microsoft Office Excel 2007 (Microsoft Corporation, 2007). Statistical analysis was done as described in section 3.2.2 of chapter 3.and exported to SPSS and GENSTAT for Windows Edition 2 (VSN International). Parameters between the first and

second visits to the clinic were compared using Independent t-test to check for significant differences at a confidence interval of 95% (p≤0.05).

4.3 Results

4.3.1 Signalment

Of the 192 dogs recruited into the study there were 61% (117/192) males and 39% (75/192) females infected with ehrlichia based on the clinical and microscopic examination during the study period. The German shepherd breed was over-represented with 51% (98/192) of all the ehrlichia infected dogs. Table 3 presents data on the distribution of ehrlichiosis among the various breeds. Cross-breeds were 16.7 % (32/192), GSD crosses 6.3% (12/192), Rottweilers 4.2% (8/192), Labradors 3.7% (7/192), Ridgebacks 2.6% (5/192), Dobermans 1.7% (3/192) and Japanese spitzs 0.5 (1/192). Indigenous breeds were 4.2% (8/192) and nondescript dogs were 9.4% (18/192).

Table 3. Different breeds diagnosed with ehrlichiosis.

Dog breed	Percentage
German Shepherd	51 (98/192)
Labrador	3.7(7/192)
Rottweiler	4.2 (8/192)
Ridgeback	2.6 (5/192)
Doberman	1.6 (3/192)
Spitz	0.5 (1/192)
Cross breeds	16.7 (32/192)
German Shepherd crosses	6.3 (12/192)
Local	4.2 (8/192)
Nondescript dogs*	9.4 (18/192)

* Nondescript dogs: dogs whose breed could not be ascertained.

4.3.2 Clinical signs

The most common clinical signs in the 192 dogs in this study were lymphadenomegaly (65.3%), congestion of mucous membranes (55.8%), panting (47%), inappetence (45.4%), wasting (33%), lethargy (31.5%), loose hair (31%), a pounding heart (26.1%), ocular discharges (22.6%), harsh lungs (20.4%) and pale mucous membranes (16.4%). The clinical signs observed during examination are presented in Table 4 and Figures 1, 2, 3 and 4.

External parasites found on these dogs were fleas in 15.7% (23/147) and ticks in 17.2% (25/145).

In most dogs, the lymphadenopathy was generalized with submandibular lymph node being the commonest affected one. A large number of dogs diagnosed with these infections had no changes affecting the lymph nodes (Figure 5).

Table 4. Clinical signs observed in dogs diagnosed with ehrlichiosis.

Clinical signs	Percentage
Lethargy	31.5 (51/162)
Weakness	13.2 (14/106)
Inappetance	45.4 (74/163)
Pale mucous membranes	16.4 (24/146)
Congestion of mucous membrane	55.8 (92/165)
Ocular discharge	22.6 (36/159)
Harsh lungs	20.4 (34/167)
Panting	47 (62/132)
Loose hair	31 (49/158)
Vomiting	12.2 (16/131)
Wasting	33 (54/164)
Splenomegaly	9.3 (15/162)
Lymphadenomegaly	65.3 (89/161)
Tender abdomen	15.4 (25/162)
Diarrhoea	16 (21/131)
Haemorrhage	13.4 (22/164)
Hind limb weakness	5.3 (6/114)
Pounding heart	26.1 (42/161)
Scleral injection	9.6 (9/94)
Icterus	1.9 (2/107)
Nasal discharge	2.8 (3/106)
Flea allergy dermatitis	1.8 (3/165)
Dermatitis	12.2 (17/139)

Figure 1. A dog naturally infected by Ehrlichia with wasting during the first visit for management.

Figure 2. Front view of the naturally infected dog showing wasting of the muscles of the head, scapula spine and prominent hip bones.
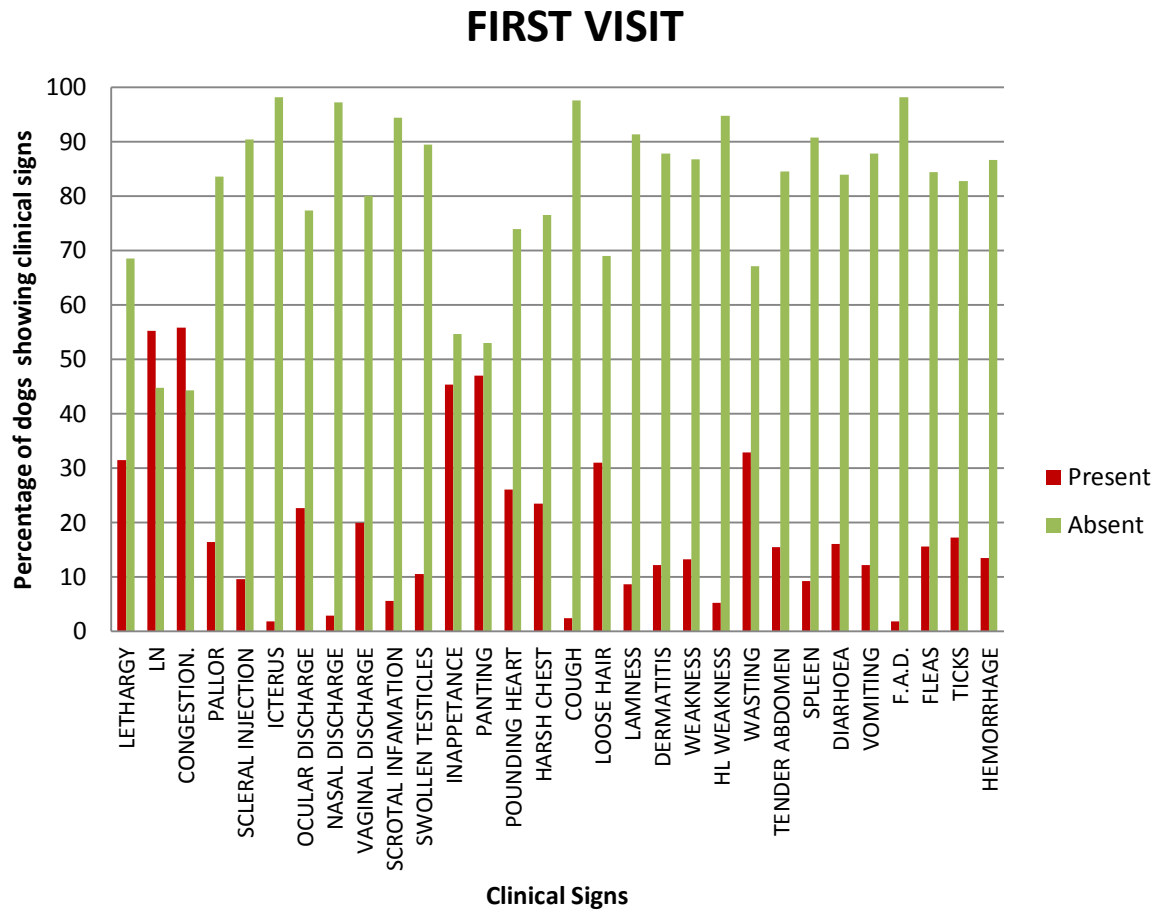
FIRST VISIT

Figure 3. Percentage of dogs showing clinical signs observed in dogs diagnosed with ehrlichiosis during the first visit.

LN =Lymph nodes
F.A.D.= Flea allergic dermatitis

SECOND VISIT
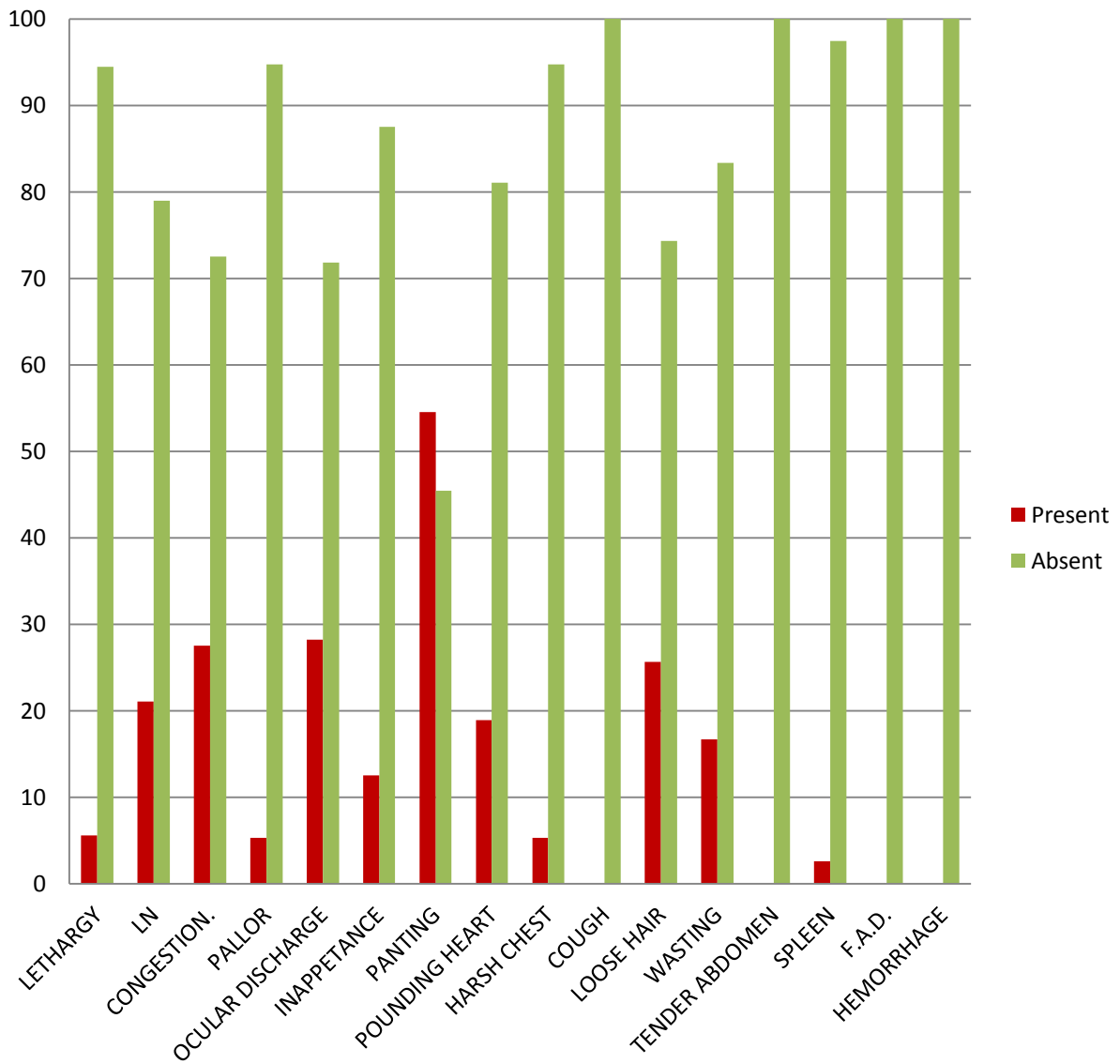
Figure 4. Percentage of dogs showing clinical signs of ehrlichiosis observed during the second visit.

LN =Lymph nodes
F.A.D.= Flea allergic dermatitis
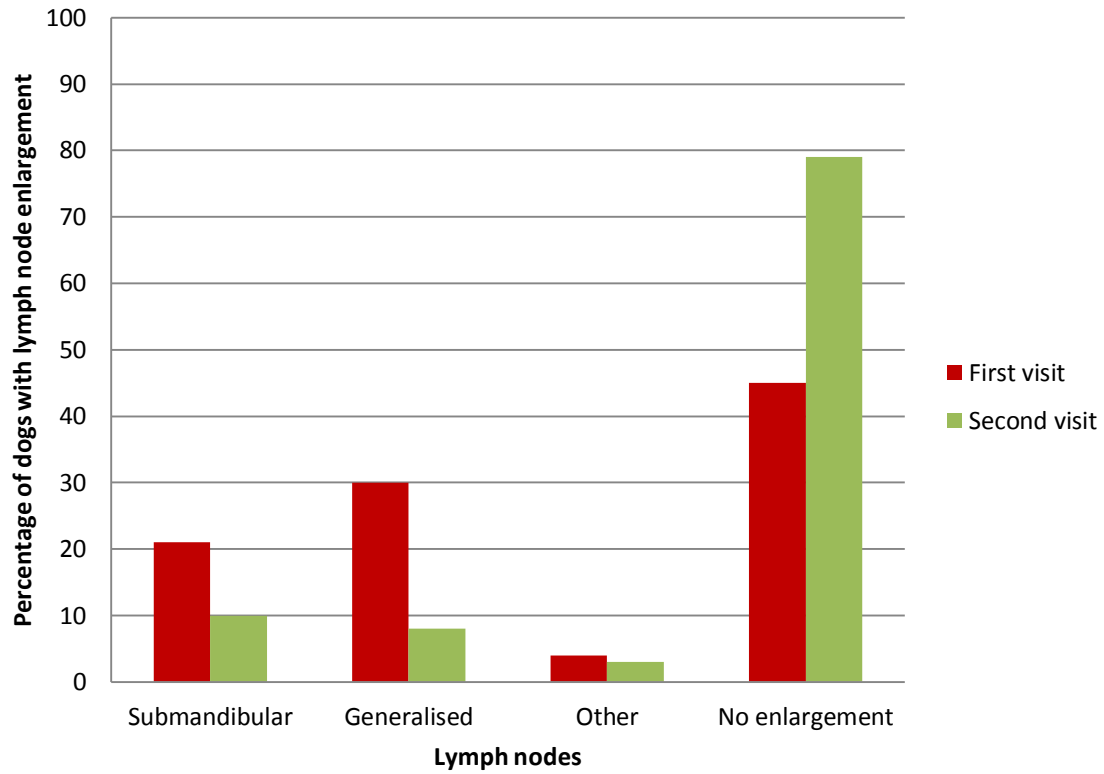
Figure 5. Percentage of dogs showing change in lymph nodes size observed in dogs diagnosed with ehrlichiosis during the first and second visit.

Microscopic examination of Giemsa stained blood smears revealed the presence of intracytoplamic inclusion bodies in the nucleated cells. Most of the inclusion bodies were noted in monocytes (Figure 6 and 7). Only a few cases had inclusion bodies in the polymorphonuclear cells (Figure 8). The microscopic examination showed 67% (129/192) of the samples with inclusions in the monocytes and lymphocytes, 21% (40/192) in granulocytes and 12% (23/192) in both the mononuclear and polymorphonuclear cells (Figure 9).

Figure 6. A blood smear from one of the cases in the study showing several inclusion bodies in a monocyte (Arrows).
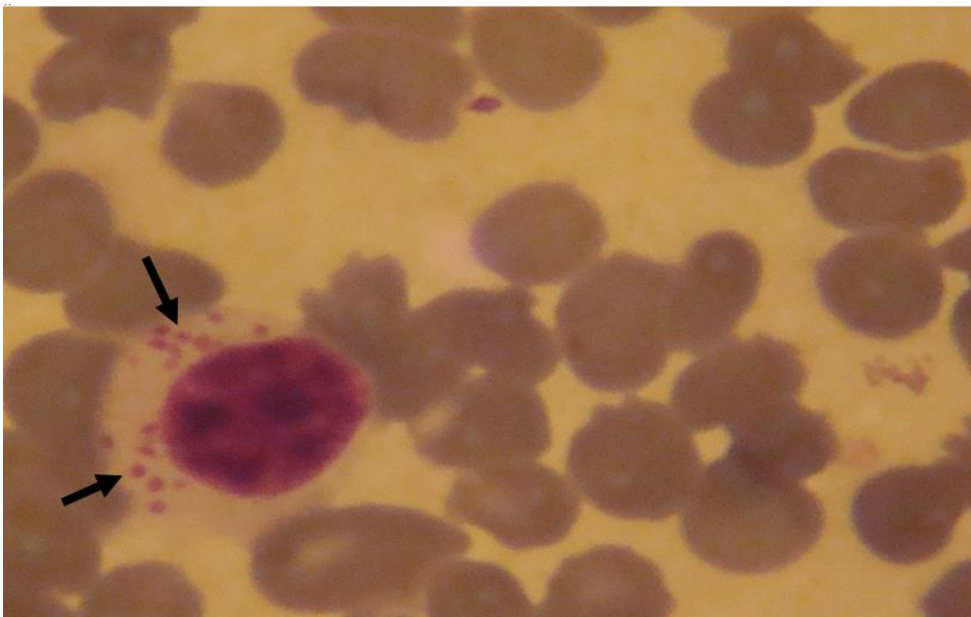
Figure 7. A blood smear from one of the cases in the study showing numerous inclusion bodies in a monocyte (Arrows).

Figure 8.. A blood smear from one of the dogs in the study showing a morula (arrow) in multinucleated white blood cell.
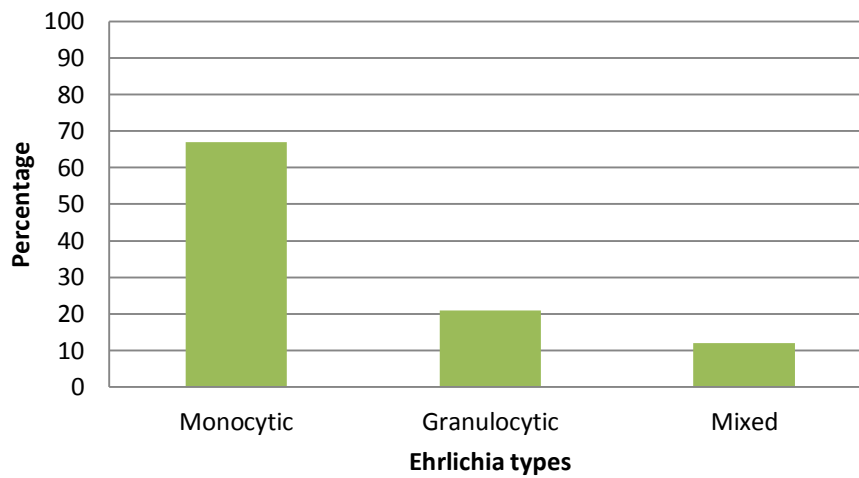
Figure 9. Percentage of dogs diagnosed with particular the types of ehrlichia inclusion bodies observed in blood smears.

4.3.3. Haematology

Haematological analysis revealed significant ($p<0.05$) changes between the first visit and the second visit by the dogs in mean (mean ±std error of mean) lymphocytes counts from 27.22±0.94 to 30.72±1.4. This increase was mainly caused by mature lymphocytes which increased from the mean value of 24.28±1.03 to 29.38±1.91 ($p<0.01$) (Table 5). On the other hand, granulocyte mean value decreased significantly ($p<0.05$) from 72.34±1.1in the first visit to 67.14±2.01 in the second visit. This was mainly due to the decrease in neutrophils whose mean value decreased from 66.72±1.05 to 61.65±1.38 ($p<0.01$). There was an increase in the mean thrombocyte count between the first and second visit from 197.03±11.99 to 209.64±19.58 though this was not significant ($p=0.58$).

There was a slight increase in mean red blood cells count from 6.02±0.14 to 6.46±0.19 ($p=0.091$). The haematocrit, however, showed a significant ($p<0.05$) increase from 40.83±0.99 to 44.51±1.17.The mean haemoglobin concentration increased minimally from 13.1±0.26 to 13.87±0.31 ($p=0.096$). The MCH and MCHC both showed a non-significant decrease in the mean values of 23.26±0.83 to 21.31±0.51 ($p=0.149$) and 33.49±0.79 to 31.54±0.68 ($p=0.14$) respectively (Table 5).

For the Imidocarb dipropionate treated dogs, changes in mean values between the first and second visits were not significant except for the MCHC whose mean value increased from 32.15±0.9 to 35.95±0.52 ($p<0.05$) while the MCH increased from 21.61±0.67 to 24.2±0.64 ($p=0.09$). The mean haemoglobin concentration showed an increase from

12.99±0.4 to 14.84±0.93 (p=0.06) which, however, was not significant (p>0.05) (Table 6).

4.3.4. Biochemistry

The mean albumin levels increased significantly (p<0.05) from 2.95±0.12 in the first visit to 3.5±0.20 in the second visit. The mean calcium levels also increased significantly (p<0.05) from 10.43±0.49in the first visit to 12.31±0.63 in the second visit. The levels of phosphorus increased from 5.47±0.35 to 19.49±14.13. However, this was not significant (p=0.207). The mean values for alkaline phosphatase and alanine aminotransferase decreased, though not significantly, from 177.7±19.09 to 129.1±27.15 (p=0.163) and 39.33±3.14 to 33.4±2.2 (p=0.243) respectively (Table 5).

The only significant (p<0.05) change noted in the evaluated biochemical parameters for the Imidocarb dipropionate treated dogs was an increase in the mean albumin levels from 2.96±0.19 to 4.02±0.49 (Table 6). Though the alkaline phosphatase levels decreased from 171.87±30.04 to 91.4±14.62, this was not significant (p=0.23).

Table 5. The means and standard errors at 95% confidence interval of the haematologic and biochemical parameters of Ehrlichia infected dogs (n=192) during the first (1) and second (2) visits.

	Visit	Mean	Std. Error Mean	Sig. at 95% CI		Visit	Mean	Std. Error Mean	Sig. at 95% CI
HR	1	98.69	± 2.43		**MCHC**	1	33.49	± 0.79	
	2	95.45	± 5.26	.589		2	31.54	± 0.68	.140
RR	1	36.67	± 2.12		**RDW**	1	11.32	± 0.15	
	2	38.25	± 3.71	.752		2	11.22	± 0.20	.734
TEMP	1	39.21	± 0.06		**HB**	1	13.10	± 0.26	
	2	38.98	± 0.10	.107		2	13.87	± 0.31	.096
WBC	1	13528.95	± 632.63		**THROMBO**	1	197.03	± 11.99	
	2	11858.09	± 571.01	.126		2	209.64	± 19.58	.577
LYMP	1	27.22	± 0.94		**MPV**	1	9.06	± 0.05	
	2	30.72	± 1.40	.050		2	9.03	± 0.07	.747
LYMPHM	1	24.28	± 1.03		**PCT**	1	0.18	± 0.01	
	2	29.38	± 1.91	.013		2	0.42	± 0.23	.105
MONO	1	0.21	± 0.06		**PDW**	1	8.40	± 0.23	
	2	0.16	± 0.08	.585		2	8.93	± 0.15	.167
MONOCYM	1	3.29	± 0.12		**BUN**	1	19.43	± 1.62	
	2	3.48	± 0.15	.367		2	19.04	± 1.54	.887
GRANUL	1	72.34	± 1.10		**CREATININE**	1	2.01	± 0.97	
	2	67.14	± 2.01	.017		2	1.07	± 0.06	.533
NEUTRO	1	66.72	± 1.05		**TP**	1	7.44	± 0.20	
	2	61.65	± 1.38	.009		2	7.86	± 0.30	.251
NEUTROM	1	64.28	± 1.29		**ALBUMIN**	1	2.95	± 0.12	
	2	60.33	± 1.44	.082		2	3.50	± 0.20	.018
NEUTROIMM	1	1.32	± 0.11		**GLOBULIN**	1	4.48	± 0.18	
	2	1.28	± 0.15	.861		2	4.33	± 0.31	.656
EOSINO	1	5.82	± 0.59		**A/GRATIO**	1	1.31	± 0.47	
	2	7.36	± 0.76	.150		2	1.34	± 0.28	.970
BASO	1	0.02	± 0.01		**CALCIUM**	1	10.43	± 0.49	
	2	0.00	± 0.00	.363		2	12.31	± 0.63	.021
RBC	1	6.02	± 0.14		**PHOSPHORUS**	1	5.47	± 0.35	
	2	6.46	± 0.19	.092		2	19.49	± 14.13	.207
MCV	1	67.64	± 0.50		**AP**	1	177.70	± 19.09	
	2	68.24	± 0.51	.489		2	129.11	± 27.15	.163
HCT	1	40.83	± 0.99		**ALT**	1	39.33	± 3.14	
	2	44.51	± 1.17	.036		2	33.40	± 2.20	.243
MCH	1	23.26	± 0.83						
	2	21.31	± 0.51	.149					

Table 6. The means and standard errors at 95% confidence interval of the haematologic and biochemical parameters of Imidocarb dipropionate treated dogs during the first (1) and second (2) visits.

	Visit	Mean	Std. Error Mean	Sig. at 95% CI
HR	1	102.76	± 3.40	
	2	86.00	± 6.63	0.20
RR	1	34.40	± 1.77	
	2	32.00	± 0.00	0.72
TEMP	1	39.21	± 0.08	
	2	39.17	± 0.21	0.90
WBC	1	14392.34	± 1103.10	
	2	13020.00	± 1555.46	0.59
LYMP	1	27.33	± 1.51	
	2	25.11	± 1.34	0.54
LYMPHM	1	23.54	± 1.63	
	2	20.39	± 1.57	0.38
MONO	1	0.26	± 0.10	
	2	0.00	± 0.00	0.24
MONOCYM	1	3.10	± 0.17	
	2	2.66	± 0.23	0.23
GRANUL	1	73.31	± 1.75	
	2	76.96	± 1.72	0.35
NEUTRO	1	65.33	± 1.57	
	2	64.67	± 2.05	0.86
NEUTROM	1	62.39	± 2.25	
	2	63.13	± 2.57	0.89
NEUTROIMM	1	1.25	± 0.16	
	2	1.75	± 0.37	0.23
EOSINO	1	6.69	± 1.04	
	2	10.22	± 1.46	0.17
BASO	1	0.02	± 0.02	
	2	0.00	± 0.00	0.65
RBC	1	6.17	± 0.24	
	2	6.10	± 0.34	0.90
MCV	1	67.58	± 0.67	
	2	67.56	± 1.46	0.99
HCT	1	42.03	± 1.72	
	2	41.27	± 2.61	0.84
MCH	1	21.61	± 0.67	
	2	24.20	± 0.61	0.09

	Visit	Mean	Std. Error Mean	Sig. at 95% CI
MCHC	1	32.15	± 0.90	
	2	35.95	± 0.52	0.05
RDW	1	11.47	± 0.26	
	2	10.99	± 0.65	0.45
HB	1	12.99	± 0.40	
	2	14.84	± 0.93	0.06
THROMBO	1	197.18	± 18.03	
	2	141.56	± 18.54	0.16
MPV	1	9.08	± 0.08	
	2	9.00	± 0.12	0.67
PCT	1	0.18	± 0.02	
	2	0.13	± 0.02	0.16
PDW	1	8.45	± 0.42	
	2	9.24	± 0.35	0.39
BUN	1	18.79	± 3.51	
	2	20.09	± 2.64	0.87
CREATININE	1	1.01	± 0.06	
	2	1.04	± 0.12	0.80
TP	1	7.31	± 0.35	
	2	7.64	± 0.75	0.68
ALBUMINE	1	2.96	± 0.19	
	2	4.02	± 0.49	0.03
GLOBULINE	1	4.43	± 0.33	
	2	4.02	± 0.70	0.59
AGRATIO	1	2.05	± 1.27	
	2	1.52	± 0.48	0.84
CALCIUM	1	10.59	± 0.78	
	2	11.84	± 1.39	0.41
PHOSPHORUS	1	5.66	± 0.62	
	2	7.33	± 1.31	0.20
AP	1	171.87	± 30.04	
	2	91.40	± 14.62	0.23
ALT	1	31.16	± 3.70	
	2	35.30	± 5.39	0.62

4.3.5. Treatment and outcome

The dogs were treated with Imidocarb dipropionate at a dose of 5 mg/kg body weight intramuscularly58.9% (86/146), oral doxycycline at a dose of 10 mg/kg body weight8.9% (13/146), or a combination of Imidocarb dipropionate with doxycycline 32.2% (47/146). General improvement was noted in the treated dogs that were presented for follow up by the owners. This was reflected by a reduction in the percentage of dogs showing clinical signs on the second visit. Some of the clinical signs reported on the first visit (Figures 3) were not observed on the second visit (Figures 4).

Lymphadenopathy that had been observed in the dogs had resolved or reduced and the percentage of animals with no enlargement of lymph nodes had increased by the second visit (Figure 5). Some of the clinical signs observed during the first visit such as tender abdomen, splenomegaly and haemorrhage had completely resolved while the percentage of dogs noted with the other clinical signs had remarkably reduced (Figures3 and 4).

4.4. Discussion

The clinical indication of anaemia, pale mucous membranes, observed in 16.7% of dogs with ehrlichial infection have also been reported in cases of *Ehrlichia* and *Anaplasma* infections (Neer, 1995; Harrus *et al*. 1997a; Bexfield *et al*. 2005). Examination of dogs 14 days post treatment, revealed that haematologic parameters red blood cells, packed cell volume and haemoglobin concentration increased. However, only the increase in packed cell volume was statistically significant ($p<0.05$). The increases are an indication of possible resolution of anaemia in these animals. These increases are an indicator to the

fact the dogs were responding to therapy and recovering from the effects of ehrlichial infection. In addition, it can be taken to be a prognostic indicator of recovery. This observation is supported by a previous report by Shipov *et al*. (2008) which indicated that platelet counts above 89.5×10^3 µL and PCV above 33.5% were among findings that predicted survival in dogs suffering from canine monocytic ehrlichiosis. Decreases in haematocrit, erythrocyte count and haemoglobin concentration have been reported in animals suffering from ehrlichiosis (Al-Badrain, 2013; Purnell *et al*. 1977; Buhles *et al*. 1975). This may be the result of an increased rate of destruction or impaired erythropoiesis during the infection.

Though Farias Rotondano *et al*. (2012) reported anaemia in 26.6% of cases in a study; the results demonstrated that thrombocytopenia is not sufficient to diagnose either ehrlichiosis or anaplasmosis. Santos *et al*. (2009) also observed a high incidence of *E. canis* infection among nonthrombocytopenic dogs. Moreover, other diseases such as immune-mediated thrombocytopenia, neoplasia, inflammatory diseases and other infectious agents can provoke thrombocytopenia (Grindem *et al*. 2002). Infectious canine cyclic thrombocytopenia caused by *Anaplasma platys* was not diagnosed in any dog evaluated during this study. This may be due to the fact that identification of *Anaplasma platys* morulae or inclusion bodies requires a systematic, careful and time consuming evaluation of blood smears. This together with the cyclic appearance of the parasite in blood makes the diagnosis a challenge to veterinary practitioners. Moreover, *Anaplasma platys* infection does not generally cause clinical infection in most dogs. The only

evidence of infection in dogs may be exhibiting prolonged bleeding time in some dogs after venepuncture or from the site of tick removal (Brown *et al*. 2001).

The thrombocytopenia observed in this study was consistent with previous reports (Neer *et al*. 2002; Egenvall *et al*. 1997; Harrus *et al*.1997a &1997b) though this was not statistically significant. This may be due to the cyclic nature of thrombocytopenia that occurs with *Anaplasma platys* infection (Harvey *et al*. 1978; Stiles, 2000), if this infection was present.

Leukopenia, thrombocytopenia and anaemia of animals infected with ehrlichiosis have been attributed to possible suppression of bone marrow production (Loverin *et al*. 1980; Greig *et al*. 1977). Alternatively to the autoimmune destruction of infected leukocytes, platelets and perhaps red blood cells (Al-Badrani, 2013).

No changes were noted in the lymphatic system in a large number of dogs diagnosed with ehrlichiosis. Changes noted included enlargement of sub-mandibular lymph nodes though, in most dogs generalized enlargement of lymph nodes occurred. Lymphadenopathy has been reported as one of the clinical presentations in dogs infected with Ehrlichia and Anaplasma (Waner and Harrus, 2000; Harrus *et al*. 1997a & 1997b).

A notable finding in this study was congestion of conjunctival mucous membranes. Congestion of mucous membranes was also reported by Price (1980) in naturally occurring cases of acute ehrlichiosis. However, no reference was made to the same

clinical observation on experimental studies of the infection. In this study, congestion of conjunctival mucous membranes was observed in 55.4% of the cases presented during the first visit which reduced to 27.7% 14 days post-treatment. Congestion of the conjunctival mucous membranes may, therefore, be an important clinical observation in the tentative diagnosis of natural canine ehrlichiosis in the tropics.

CHAPTER 5

5.0 Molecular characterization of canine ehrlichiosis and anaplasmosis in Nairobi, Kenya

5.1 Introduction

Ehrlichiae and Anaplasma are important, emerging tick-borne gram negative, obligate intracellular bacteria from the *Anaplasmataceae* family that infect canines and are also zoonotic (Stuen, 2007; Woldehiwet, 2006; Greig *et al*. 1996). In the host cells, monocytes and granulocytes, the bacteria reside in inclusion bodies, the morulae. Dogs maybe infected by several Anaplasmataceae agents such as *Ehrlichia canis, E. ewingii, E. chaffeensis, Anaplasma platys*, *A. phagocytophilum* and *Neorickettsia risticii* (Unver *et al*. 2001a; Kordick *et al*. 1999; Breitschwerdt *et al*. 1998a; Goldman *et al*. 1998; Murphy *et al*., 1998; Dawnson *et al*. 1996).

Polymerase Chain Reaction (PCR) is a method widely used for the laboratory diagnosis of infectious diseases (Anderson *et al.,* 1991), such as canine monocytic ehrlichiosis, especially during the acute phase of the disease before antibodies are detectable (Wen *et al*. 1997). Alexandre *et al*., (2009) reported detection by a nested PCR of *Ehrlichia canis* in dogs that were seronegative by indirect IFA test. Several studies have shown PCR to be an effective and extremely sensitive method for the detection of *Ehrlichia* and *Anaplasma* species in dog blood and tissues (Dawson *et al.* 1996; Engvall *et al*. 1996; McBride *et al*. 1996; Iqbal and Rikihisa 1994a; Iqbal *et al*. 1994). Chang and Pan (1996) demonstrated increased sensitivity of a nested PCR in amplification of *Anaplasma platys* from experimentally infected dogs.

Kordick *et al*. (1999) reported that 56% of dogs in a kennel in North Carolina were *E. canis* positive as determined by PCR. Murphy *et al*. (1998) detected *Ehrlichia canis* DNA in 3% of dogs in Oklahoma while Unver *et al*. (2001) reported that 31% of Venezuelan dogs were positive by PCR specific to *Ehrlichia canis*. The high infection rate demonstrated that *Ehrlichia canis* is an important pathogen among dogs in these locations, and requires attention of veterinary and public health professionals. In Kenya, confirmatory diagnosis of ehrlichial infection has been reported using indirect fluorescent antibody test, cell culture and serology (Alexander *et al*. 1994; Price, 1980; Kaminjolo *et al*. 1976). However, these tests focused on *Ehrlichia canis;* reports on diagnosis of other species of *Ehrlichia* and *Anaplasma* in dog in Kenya are scant. Molecular typing of the etiological agent for ehrlichial infections in Kenya was therefore necessary to elucidate their pathogenicity in companion animals and determine their zoonotic potential. This study was designed to determine the molecular identity of the ehrlichia organisms present in dogs in Kenya

Seroprevalence studies have reported the presence of *Ehrlichia canis* or related species infecting dogs throughout Africa (Kelly *et al*. 2004; Pretorius and Kelly, 1998; Bostros *et al*. 1995; Brouqui *et al*. 1991). Studies by Ndip *et al*. 2005 in Cameroonian canines reported the presence of *Ehrlichia canis* and *Ehrlichia ewingii* by PCR. However, reports describing *Ehrlichia chaffeensis, Anaplasma phagocytophilum* and *Anaplasma platys* infection in Kenya were not available in published scientific literature.

Previous reports of canine ehrlichiosis in Kenya describe clinical signs in experimental and natural infection, identification of intracytoplamic inclusion bodies, cell culture and indirect fluorescent antibody test (Alexander *et al*. 1994; Price, 1980; Kaminjolo *et al*. 1976). However, there were no published scientific reports describing molecular characterization of the species of *Ehrlichia* or *Anaplasma* causing infection in dogs in Kenya. A study was therefore designed with the objective to determine the molecular characteristics of the species of ehrlichial infections, to establish the associated clinical features and to evaluate treatment.

A study to establish the species of the Anaplasmataceae agents would elucidate the full extent of their zoonotic potential and aid in understanding the clinical features, particularly where confirmatory diagnostic tools are not readily available.

5.2 Materials and Methods

5.2.1 Sample collection

Blood collection and processing for thin smears, haematology and blood chemistry analysis was performed as described in sections 4.2.1, 4.2.2 and 4.2.3 of chapter 4.

Three millilitres of blood for molecular study from dogs diagnosed with ehrlichia by use of blood smears was collected aseptically by venepuncture in plastic tubes containing the anticoagulant ethylenediaminetetraacetic acid (EDTA).

5.2.2. DNA extraction

Ethylenediaminetetraacetic (EDTA) blood for PCR was stored at -20° C until used for DNA extraction. The DNA was extracted from 200 µl of each blood specimen. The QIAmp blood and tissue extraction kit (Qiagen, Hilden, Germany) was used for DNA extractions, following the manufacturer's protocols. The DNA extraction procedure was as described below.

Procedure

1. 20 µl QIAGEN Protease was pipetted into the bottom of a 1.5 ml microcentrifuge tube.

2. 200 µl whole blood sample was then added to the 1.5 ml microcentrifuge tube.

3. 200 µl Buffer AL was added to the sample and mixed by pulse-vortexing for 15 seconds.

4. The mixture was then incubated at 56°C for 10 minutes.

5. The microcentrifuge tube was then briefly centrifuged to remove drops from the inside of the lid.

6. 200 µl ethanol (96-100%) was added to the sample, and mixed by pulse-vortexing for 15 seconds. The microcentrifuge tube was briefly centrifuged to remove drops from the inside of the lid.

7. The mixture from step 6 was then carefully applied to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. The cap was then closed, and centrifuged at 6000 x g (8000 rpm) for 1 minute. The QIAamp Mini

spin column was then placed in a clean 2 ml collection tube, and the tube containing the filtrate discarded.

8. The QIAamp Mini spin column was carefully opened and 500 µl Buffer AW1 added without wetting the rim. The cap was closed and column centrifuged at 6000 x g (8000 rpm) for 1 minute. The QIAamp Mini spin column was then placed in a clean 2 ml collection tube, and the tube containing the filtrate discarded.

9. The QIAamp Mini spin column was carefully opened and 500 µl Buffer AW2 added without wetting the rim. The cap was closed and column centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 minutes.

10. The QIAamp Mini spin column was placed in a clean 2 ml collection tube and the old tube with the filtrate discarded. The column was then centrifuged at full speed for 1 minute.

11. The QIAamp Mini spin column was then placed in a clean 1.5 microcentrifuge tube, and the collection tube containing the filtrate discarded. The QIAamp Mini spin column was then carefully opened, 200 µl Buffer AE added and Incubated at room temperature (15-25°C) for 1 minute then centrifuged at 6000 x g (8000 rpm) for 1 minute.

The extracted DNA was then stored at -20°C until used.

A nested –PCR method was established with primers deprived from the 16S rRNA gene sequences of *E. canis*, *E. chaffeensis*, *E. ewingii*, *A. platys*, and *A. phagocytophilum*. For each dog PCR amplification was performed with broad-range ehrlichia genus primers and

species-specific primers. The amplifications were done as described by Breitschwerdt *et al*. (1998a).

5.2.3 Polymerase chain reaction

The amplification of the Ehrlichia genus DNA was done using genus specific set of primers that amplify a 478 base-pair fragment from the 5' half of the 16S rRNA gene.

ECC   5'-AGAACGAACGCTGGCGGCAAGCC-3'

ECB   5'-CGTATTACCGCGGCTGCTGGC-3'

Ehrlichia species-specific primers used in the amplification for the various species were:

Ehrlichia canis

ECAN 5   5'-CAATTATTTATAGCCTCTGGCTATAGGA-3'

HE3   5'-TATAGGTACCGTCATTATCTTCCCTAT-3'

Ehrlichia chaffeensis

HE1   5'-CAATTGCTTATAACCTTTTGGTTATAAAT-3'

HE3   5'-TATAGGTACCGTCATTATCTTCCCTAT-3'

Ehrlichia ewingii

EE52   5'-CGAACAATTCCTAAATAGTCTCTGAC-3'

HE3   5'-TATAGGTACCGTCATTATCTTCCCTAT-3'

Anaplasma phagocytophilum

HGE1   5'-TCCTGGCTCAGAACGAAC-3'

706R   5'-TCCTGTTTGCTCCCCACGCTTTC-3'

Anaplasma platys

Platys   5'-GATTTTTGTCGTAGCTTGCTATG-3'

EHR16SR   5'-TAGCACTCATCGTTTACAGC-3'

The extracted DNA from each sample were then used in PCR amplifications in a thermocycler (MiniCycler[TM], MJ Research, Inc. 149 Grove Street Watertown, MA 02172 USA) with primers that amplify a portion of the 16S rRNA gene. Primer ECC (5'-AGAACGAACGCTGGCGG-   CAAGCC-3')   and   ECB   (5'-CGTATTACCGCGGCTGCTGGC-3') which amplify all Ehrlichia species (Dawson *et al*. 1996; Murphy *et al*. 1998) were used. These primers amplify a 478 base-pair fragment of the Ehrlichia 16S rRNA (Dawson *et al*. 1994)

Reaction (25 µl) contained 7.5 µl of template DNA in 12.5 µl Premix containing AmpliTaq polymerase (Qiagen), 1.25 µl of each primer and 2.5 µl distilled water. The thermocycle profile was as described below:

1) Initial denaturation 94^0C for 5 minutes

2) Denaturation 95^0C for 1 minute

3) Annealing 60^0C for 1 minute

4) Extension 72^0C for 1 minute

5) GO TO STEP 2 for 40 cycles

6) Final extension 72^0C for 5 minutes

7) Store at 4^0C for ∞

The PCR reactions that resulted in positive amplification of a segment of the *Ehrlichia* species 16S rRNA were taken through a second PCR amplification using the species specific primers.

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For *Ehrlichia canis* primers HE3 and ECANS5 were used and the template was the PCR product from the first reaction. The reaction (25 µl) contained 1.0 µl of template DNA in 12.5 µl Premix containing AmpliTaq polymerase (Qiagen), 2.5 µl of each primer and 6.5 µl distilled water. The thermocycle profile was as described below:

1) Initial denaturation 94^0C for 10 minutes

2) Denaturation 94^0C for 1 minute

3) Annealing 60^0C for 1 minute

4) Extension 72^0C for1 minute

5) GO TO STEP 2 for 40 cycles

6) Final extension 72^0C for 4 minutes

7) Store at 4^0C for ∞

For *Ehrlichia chaffeensis* primers HE1 and HE3 were used and the template was the PCR product from the first reaction. The reaction (25 µl) contained 7.5 µl of template DNA in 12.5 µl Premix containing AmpliTaq polymerase (Qiagen), 1 µl of each primer and 3.0 µl distilled water. The thermocycle profile was as described below:

1. Pre-denaturation 94^0C for 3 minutes

2. Denaturation 94^0C for 1minute

3. Annealing 45^0C for 2 minutes

4. Extension time 72^0C for 30 seconds

5. Go to Step 2 for 40 cycles

6. Final extension 1 minute

7. Store at 0^0C for ∞

For *Anaplasma platys* primers PLATYS and EHR16SR were used and the template was the PCR product from the first reaction. The reaction (25 µl) contained 1.0 µl of template DNA in 12.5 µl Premix containing AmpliTaq polymerase (Qiagen), 2.5 µl of each primer and 6.5 µl distilled water. The thermocycle profile was as described below:

1. Pre-denaturation 94^0C for 10 minutes

2. Denaturation 94^0C for 1minute

3. Annealing 60^0C for 1 minute

4. Extension 72^0C for 1 minute

5. Go to Step 2 for 40 cycles

6. Final extension 72^0C for 4 minutes

7. Store at 4^0C for ∞

All PCR products were electrophoresed through 1.3% agarose gels in Tris-boric acid-EDTA buffer, and the DNA fragments visualized by ethiduim bromide staining under UV fluorescence.

5.2.4 Statistical Analyses

The results of the clinical examinations were coded as "1" (meaning the clinical observation was present) and "0" (meaning the clinical observation was no present). All the data were stored in Microsoft Office Excel 2007 (Microsoft Corporation, 2007). The data were imported into SPSS and GENSTAT for Windows Edition 2 (VSN International). Comparisons of the parameters between the first and second visits to the clinic were done using Independent t-test to check for significant differences at a confidence interval of 95% ($p \leq 0.05$). Analysis of variance was used to check for difference among the different species.

5.3. Results

From the 192 dogs in the study PCR analysis revealed 113 (58.9%) were positive for the genera Anaplasma and Ehrlichia. From these samples, a 478 base-pair product was amplified using ECC and ECB primers (Figures 10). Forty eight (48) samples representing 42.5% of the positive for the genera yielded a 358 base pair product for the primers HE3 and ECANS5 which are species specific for *Ehrlichia canis* (Figures 11 and 12), 6 samples representing 5.3% of the genera positive samples yielded a 410 base pair product for the primers HE1 and HE3 which are species specific for *Ehrlichia chaffeensis* (Figures13 and 14) and 1.8% of the samples yielded a 400 base pair product with the primers PLATYS and EHR16SR which is species specific for *Anaplasma platys* (Figure 15).
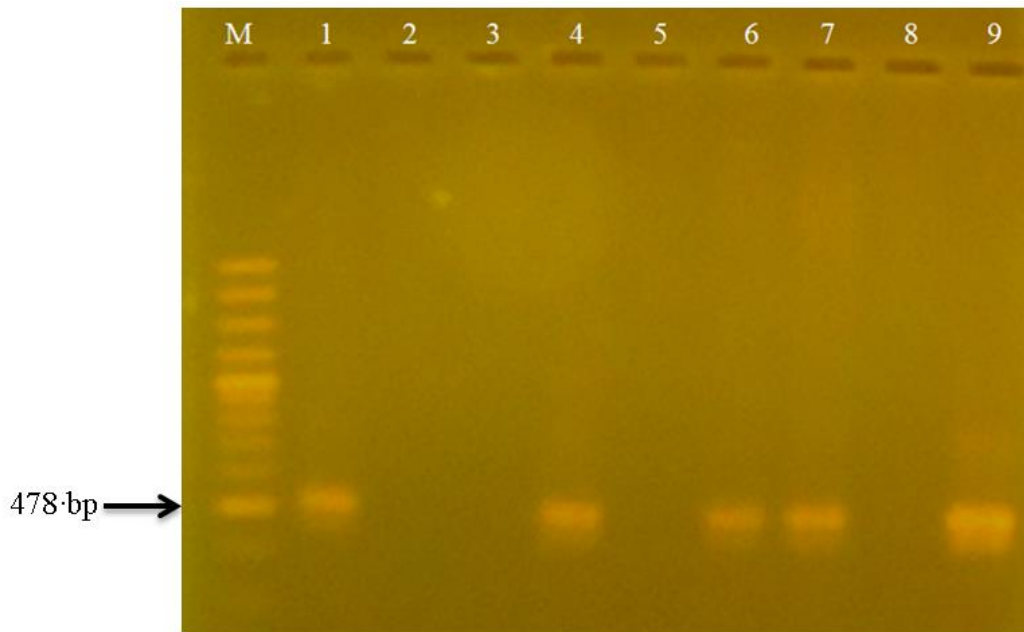
Figure 10. PCR products amplified from DNA purified from EDTA-blood samples of dogs diagnosed with ehrlichiosis using primers ECC and ECB that amplify all the *Anaplasma* and *Ehrlichia* genera.
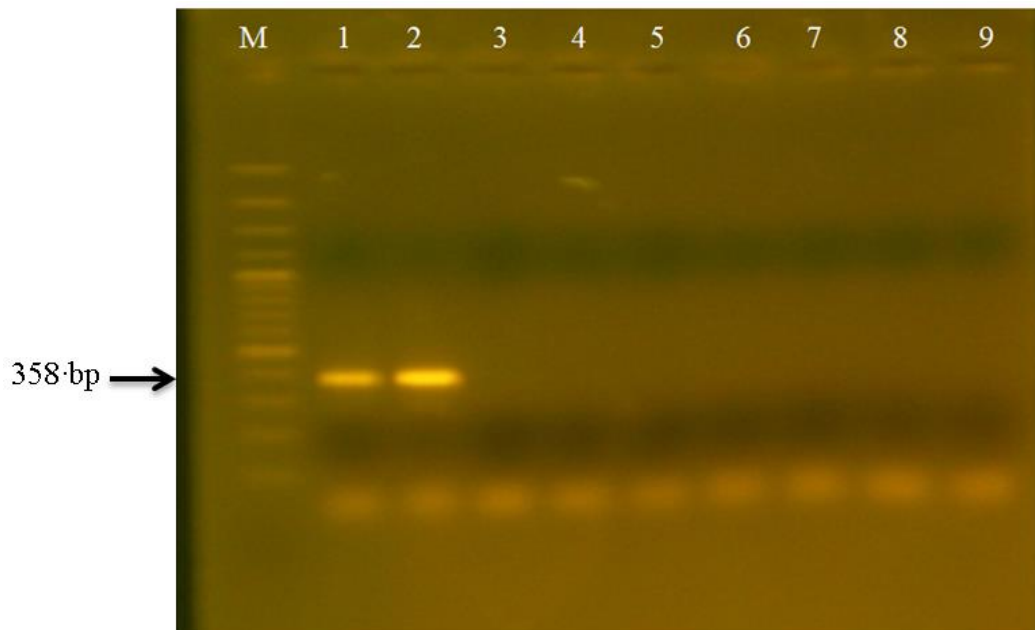
Figure 11. Nested PCR products amplified from DNA purified from EDTA-blood samples of dogs diagnosed with ehrlichiosis using primers that amplify *Ehrlichia canis*.
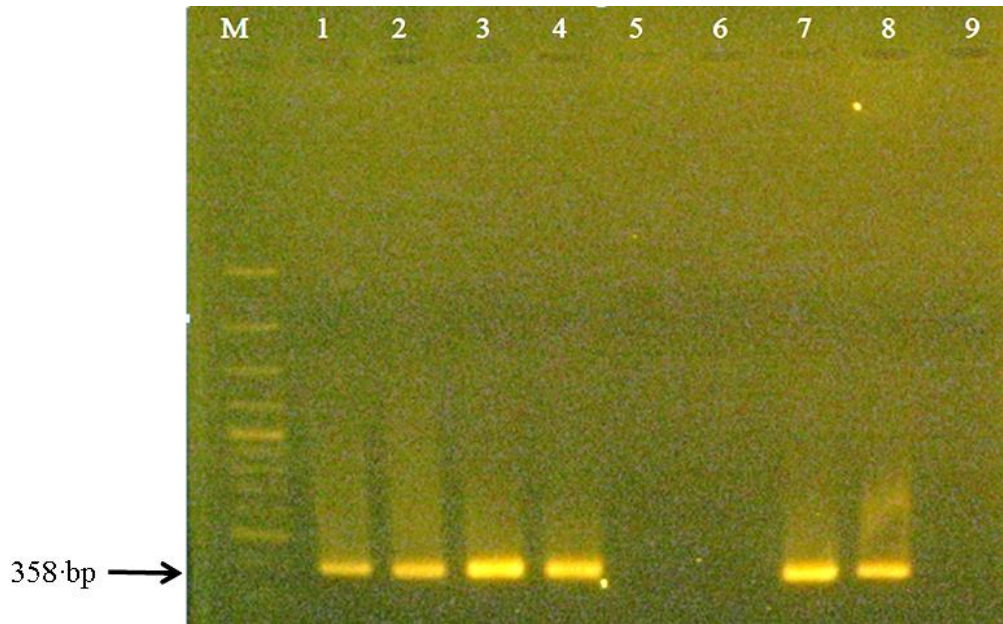
Figure 12. Nested PCR products amplified from DNA purified from another set of EDTA-blood samples of dogs diagnosed with ehrlichiosis using primers that amplify *Ehrlichia canis*.
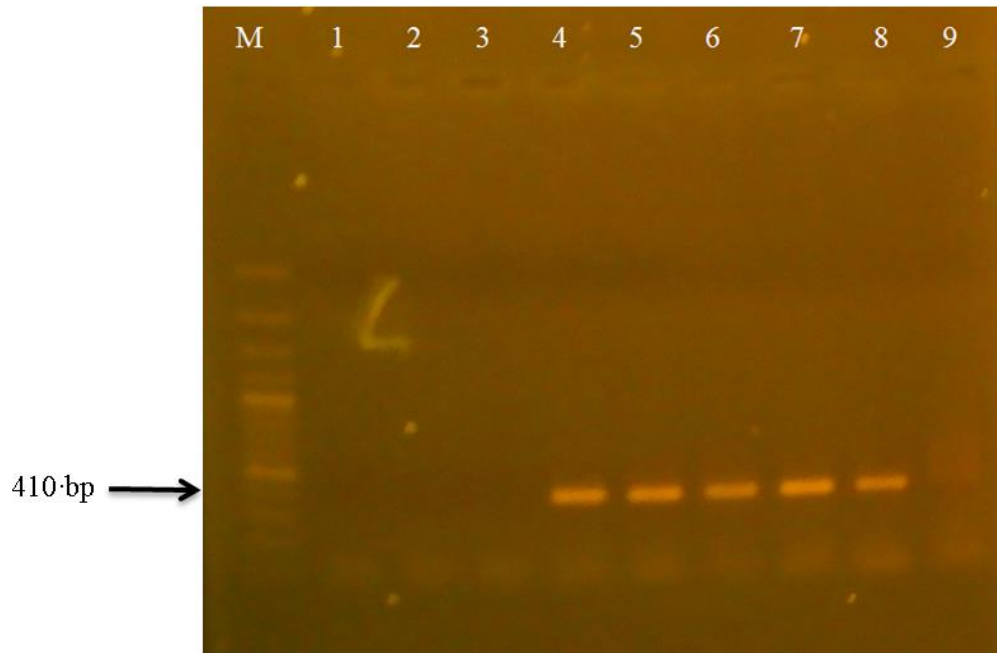
Figure 13. Nested PCR products amplified from DNA purified from EDTA-blood samples of dogs diagnosed with ehrlichiosis using primers that amplify *Ehrlichia chaffeensis*

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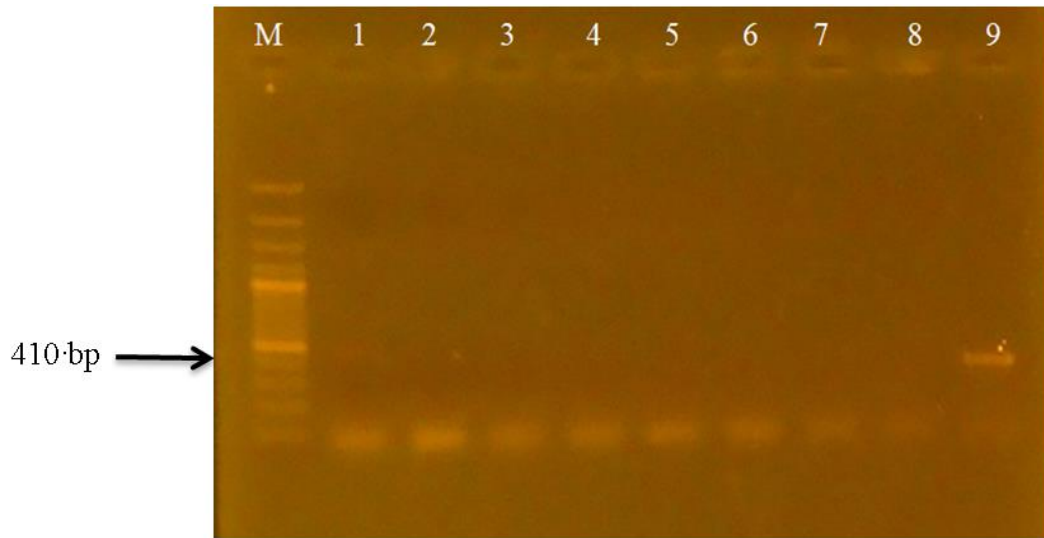
Figure 14. Nested PCR product (lane 9) amplified from DNA purified from a different set of EDTA-blood samples of dogs diagnosed with ehrlichiosis using primers that amplify *Ehrlichia chaffeensis*.
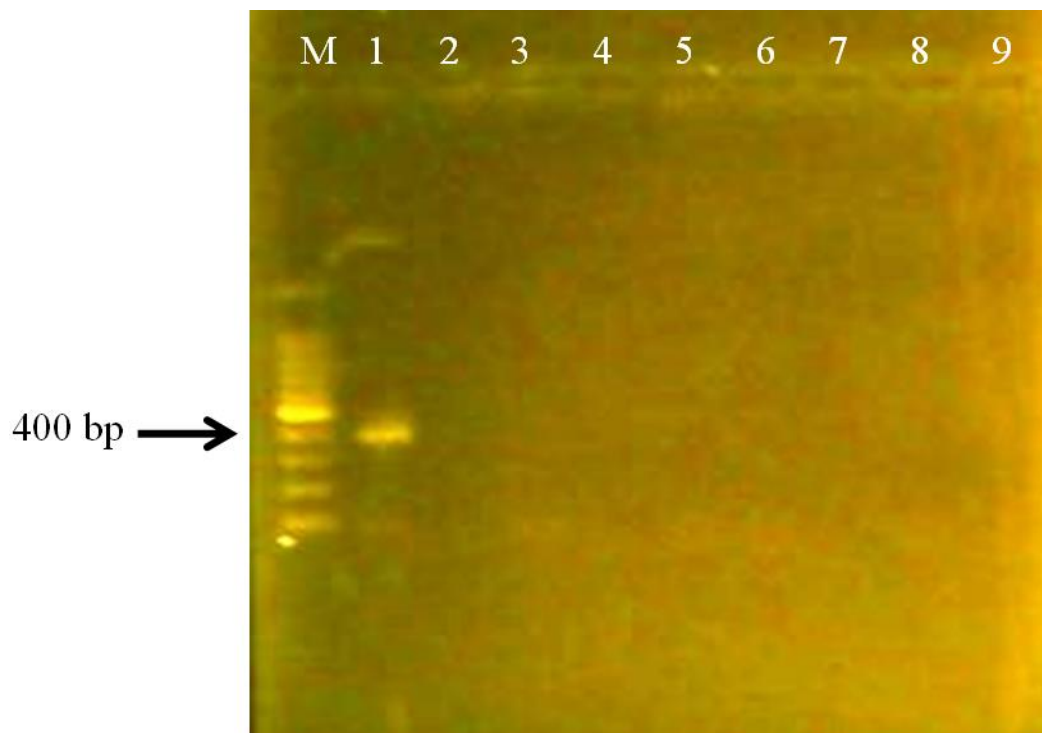
Figure 15. Nested PCR product (lane 1) amplified from DNA purified from EDTA blood of dogs diagnosed with ehrlichiosis using primers that amplify *Anaplasma platys*.

The most common clinical signs in dogs diagnosed with *Ehrlichia canis* infection by PCR were congestion of mucous membranes (53%), inappetence (49%), panting (36%), loose hair (31%), lethargy (29%),vomiting (28%), wasting (27%) and ocular discharges 27% (Table 7; Figures 16 and 17) while those in *Ehrlichia chaffeensis* and *Ehrlichia platys* are shown in Figure 18 and Figure 19 respectively.

Table 7. Clinical signs observed in dogs confirmed by PCR to be infected with *Ehrlichia canis*.

Clinical signs upon presentation of the ehrlichia positive dogs Clinical sign	Percentage
Congestion	53 (18/34)
Inappetence	49 (16/33)
Panting	36 (10/28)
Loose hair	31 (10/32)
Lethargy	29 (9/31)
Vomiting	28 (7/25)
Wasting	27 (9/33)
Ocular discharge	27 (8/24)
Diarrhoea	25 (6/24)
Pallor	18 (5/28)

There was improvement in the clinical signs reflected by drops in the percentage of dogs showing the clinical signs on the second visit to the clinic. Some of the clinical signs reported on the first visit (Figure 16) were not noted during the second visit (Figure 17).
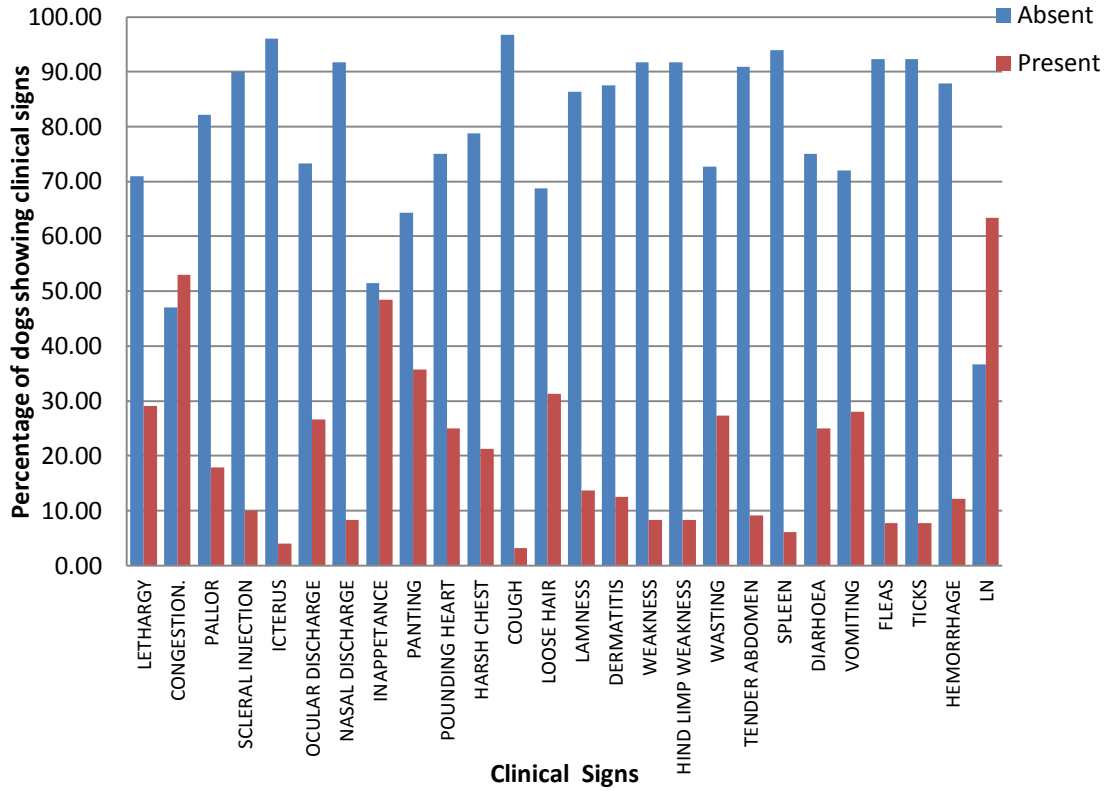
Figure 16. A bar graph showing the clinical signs observed in dogs diagnosed with *E. canis* during the first visit.
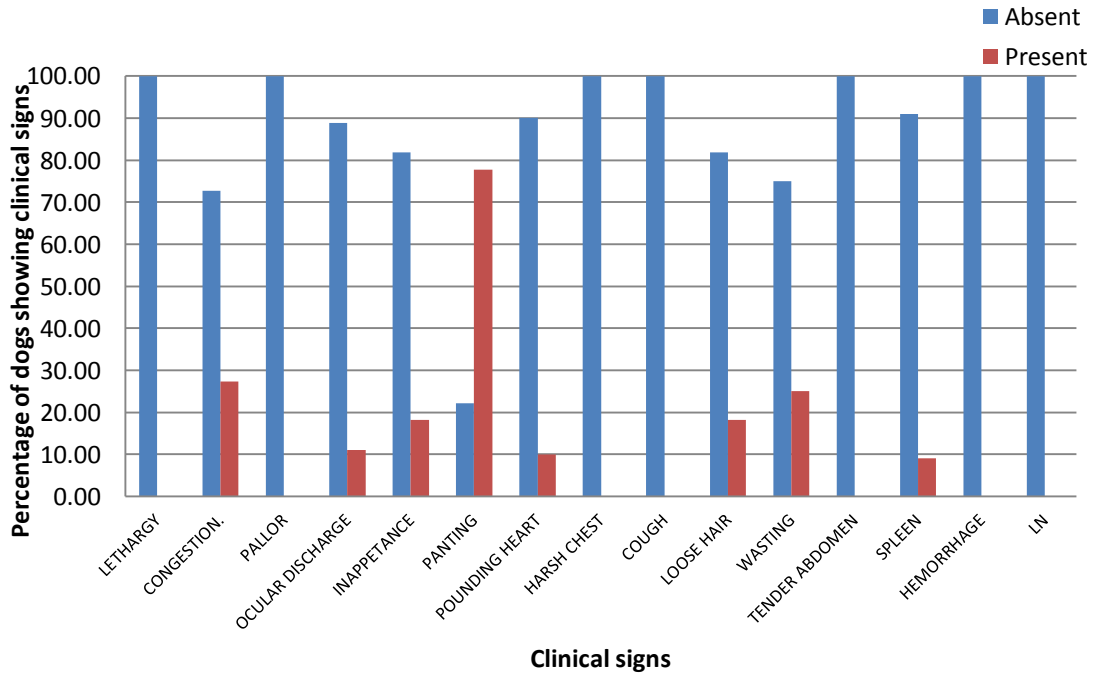
Figure 17. A bar graph showing the clinical signs observed in dogs diagnosed with *E. canis* during the second visit.
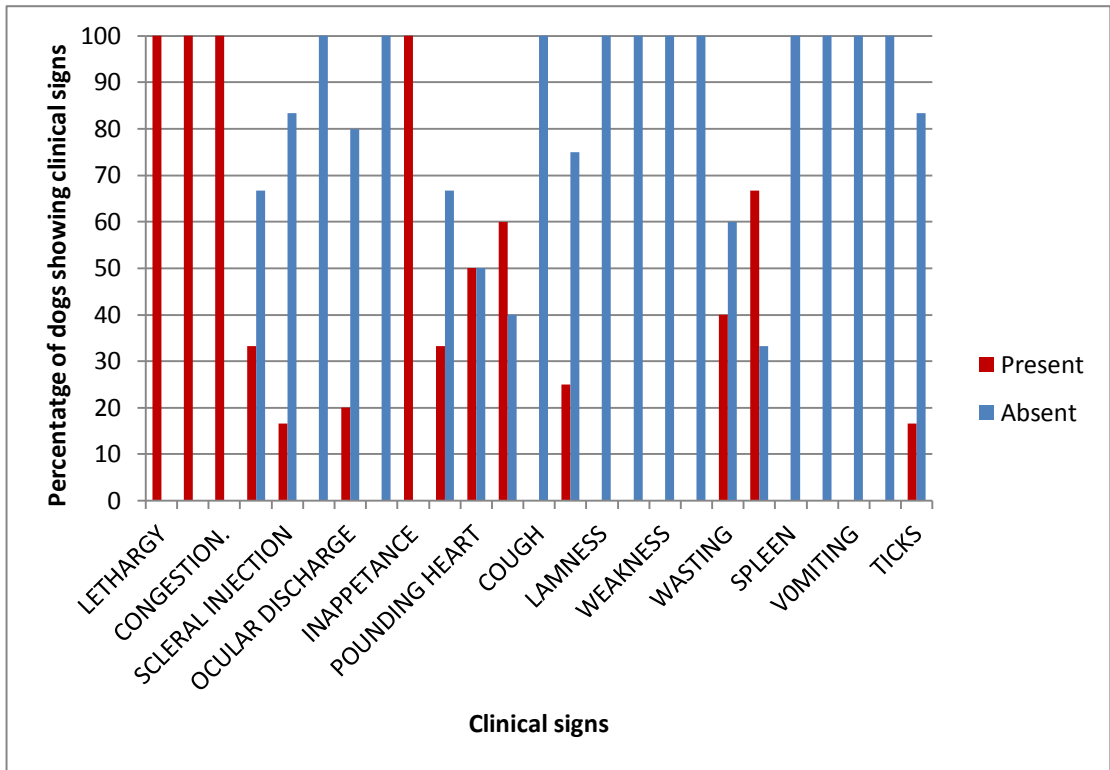
**Figure 18. A bar graph showing the clinical signs observed in dogs diagnosed with
E. chaffeensis during the first visit.**
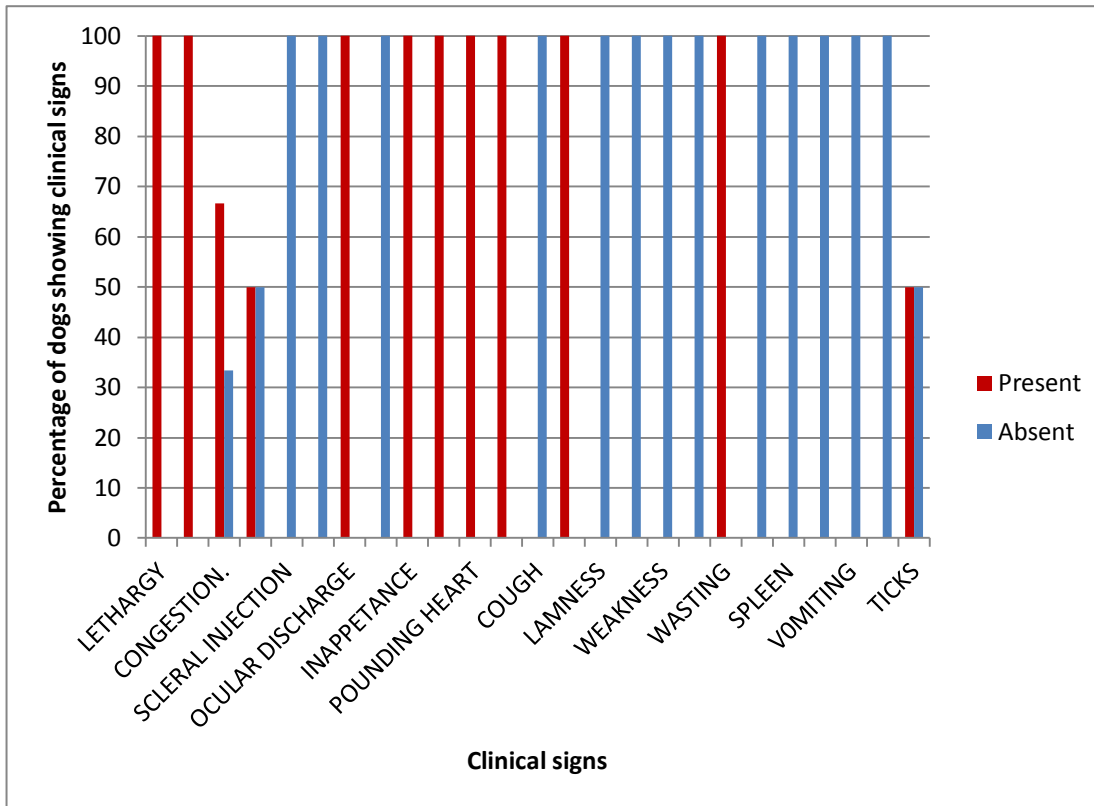
Figure 19. A bar graph showing the clinical signs observed in dogs diagnosed with A. platys during the first visit.

On presentation of the dogs the red blood cells, haematocrit and haemoglobin values were all within the reference range but on the lower side. The thrombocytes were 182,895 cells per μL which is below the reference range (Table 8). On the second visit the values had increased and were all within the reference range (Table 9). The *Ehrlichia chaffeensis* infected dogs red blood cells were $4.69x10^6$/μL, haematocrit was 33.4% and thrombocytes were 87,000 which were all below the reference ranges (Table 10). The *Anaplasma platys* infected dogs red blood cells were $4.43x10^6$/μL, haematocrit was 31.65%, haemoglobin was 11.8 g/dL and thrombocytes were 108,000 which were all below the reference ranges (Table 11).

Table 8. Values of haematological parameters of naturally infected dogs (n=48) with *E. canis* at the first visit

	Mean	Std. Deviation	Minimum	Maximum	Reference Range
WBC (cells/µL)	13392.38	5127.76	5000.00	28520.00	6000-17000
Lymphocytes (cells/µL)	3106.45	1804.49	770.00	7682.01	1000-4800
Monocytes	414.70	181.94	80.00	689.40	150-1350
Granulocytes (cells/µL)	10108.95	4551.25	4150.00	24698.32	3000-11000
RBC (X10^{6}cells/µL)	5.57	1.93	0.58	9.50	5.5-8.5
Hematocrit (%)	38.10	13.06	4.20	63.10	37-55
Hemoglobin (g/dL)	13.06	3.17	4.60	19.40	12.0-18.0
Thrombocytes (/µL)	182895	116888	30000	421000	200000-500000

Table 9. Values of haematological parameters of naturally infected dogs with *E. canis* at the second visit

	Mean	Std. Deviation	Minimum	Maximum	Reference Range
WBC (cells/µL)	12883.8	3572.0	8800.0	20270.0	6000-17000
Lymphocytes (cells/µL)	3249.0	1038.2	1571.0	4924.0	1000-4800
Monocytes	428.8	156.2	249.0	749.0	150-1350
Granulocytes (cells/µL)	9206.0	3065.3	5516.0	14878.0	3000-11000
RBC (X10^{6}cells/µL)	6.1	1.2	4.0	8.0	5.5-8.5
Hematocrit (%)	42.2	7.1	31.0	53.0	37-55
Hemoglobin (g/dL)	14.0	2.3	10.0	19.0	12.0-18.0
Thrombocytes (/µL)	230615	221105	57000	899000	200000-500000

Table 10. Values of haematological parameters of naturally infected dogs (n=6) with *E. chaffeensis* at the first visit

	Mean	Std. Deviation	Minimum	Maximum	Reference Range
WBC (cells/µL)	10143	4661	6450	15380	6000-17000
Lymphocytes (cells/µL)	2057	776	1600	2953	1000-4800
Monocytes	268	147	146	431	150-1350
Granulocytes (cells/µL)	7409	3110	4625	10766	3000-11000
RBC (X10^{6}cells/µL)	4.6867	1.17717	3.34	5.52	5.5-8.5
Haematocrit (%)	33.4	10.21029	21.9	41.4	37-55
Haemoglobin (g/dL)	12.3	4.48442	7.4	16.2	12.0-18.0
Thrombocytes (/µL)	87667	98511	16000	200000	200000-500000

Table 11.Values of haematological parameters of naturally infected dogs (n=2) with *A. platys* at the first visit

	Mean	Std. Deviation	Minimum	Maximum	Reference Range
WBC (cells/µL)	10915	6314	6450	15380	6000-17000
Lymphocytes (cells/µL)	2277	957	1600	2953	1000-4800
Monocytes	329	145	226	431	150-1350
Granulocytes (cells/µL)	8311	5212	4625	11996	3000-11000
RBC (X10^{6}cells/µL)	4.43	1.54	3.34	5.52	5.5-8.5
Hematocrit (%)	31.65	13.79	21.90	41.40	37-55
Hemoglobin (g/dL)	11.80	6.22	7.40	16.20	12.0-18.0
Thrombocytes (/µL)	108000	130108	16000	200000	200000-500000

During the first visit, the *Ehrlichia canis* infected dogs biochemical values for creatinine, total protein, albumin, globulin, calcium, and alkaline phosphatase were found to fall within the reference values. Slight increases were noted for blood urea nitrogen and phosphorus. However, there was a large increase in the value of alanine aminotransferase 59.04 U/L as compared to the reference range of 0-40 U/L (Table 12). On the second visit increases were noted for creatinine, total protein, globulin, calcium and alkaline phosphatase. The phosphorus and albumin/globulin ratio increased to beyond the reference values. The alanine aminotransferase mean serum concentration that had markedly increased on the first visit decreased to fall within the reference values (Table 13). The *Ehrlichia chaffeensis* infected dogs increased biochemistry values were noted for blood urea nitrogen 25.2 mg/dL as compared to the reference range of 10-20 U/L. Slight increase above the reference range were noted for creatinine, phosphorus and the albumin/globulin ratio. A slight decrease was however, noted in the albumin value (Table 14). The *Anaplasma platys* infected dogs had high value for blood urea nitrogen at 32.8 mg/dL and slight increases in the values of creatinine, albumin/globulin ratio and phosphorus (Table 15).

Table 12. Values of blood biochemistry parameters of naturally infected dogs (n=48) with E. canis at the first visit

	Mean	Std. Deviation	Minimum	Maximum	Reference Range
Blood Urea Nitrogen (mg/dL)	20.38	14.96	9.00	59.70	10.0-20.0
Creatinine (mg/dL)	0.99	0.35	0.40	1.80	0.6-1.2
Total Protein ((gm/dL)	7.64	2.33	4.70	12.90	5.0-8.0
Albumin (gm/dL)	3.11	1.86	1.30	8.60	2.8-4
Globulin (gm/dL)	4.37	1.48	2.30	7.20	2,7-4.4
Albumin/Globulin Ratio	1.06	1.00	0.30	4.00	0.59-1.11
Calcium (mg/dL)	9.43	2.46	5.50	15.40	8.8-10.3
Phosphorus (mg/dL)	5.49	3.84	1.70	14.50	2.5-5
Alkaline Phosphatase (U/L)	109.93	107.03	9.00	388.00	30-150
Alanine Aminotransferase (U/L)	59.04	59.36	0.00	173.50	0-40

Table 13. Values of blood biochemistry parameters of naturally infected dogs with *E. canis* at the second visit

	Mean	Std. Deviation	Minimum	Maximum	Reference Range
Blood Urea Nitrogen (mg/dL)	18.9929	12.28510	5.00	50.90	10.0-20.0
Creatinine (mg/dL)	1.2857	.44697	.80	2.40	0.6-1.2
Total Protein ((gm/dL)	8.4462	2.69803	5.40	13.80	5.0-8.0
Albumin (gm/dL)	3.6077	1.72746	1.20	7.90	2.8-4
Globulin (gm/dL)	4.8231	2.35696	.80	8.50	2,7-4.4
Albumin/Globulin Ratio	1.4285	2.56730	.30	9.90	0.59-1.11
Calcium (mg/dL)	11.4636	3.00042	8.80	18.40	8.8-10.3
Phosphorus (mg/dL)	50.4167	154.85400	1.30	542.00	2.5-5
Alkaline Phosphatase (U/L)	133.7143	214.26527	5.00	830.00	30-150
Alanine Aminotransferase (U/L)	39.0154	18.70690	3.80	68.90	0-40

Table 14. Values of blood biochemistry parameters of naturally infected dogs (n=6) with *E. chaffeensis* at the first visit

	Mean	Std. Deviation	Minimum	Maximum	Reference Range
Blood Urea Nitrogen (mg/dL)	25.20	17.41	10.00	44.20	10.0-20.0
Creatinine (mg/dL)	1.35	0.92	0.70	2.00	0.6-1.2
Total Protein ((gm/dL)	6.30	0.52	5.70	6.60	5.0-8.0
Albumin (gm/dL)	2.67	1.36	1.60	4.20	2.8-4
Globulin (gm/dL)	3.57	1.83	1.50	5.00	2,7-4.4
Albumin/Globulin Ratio	1.20	1.39	0.30	2.80	0.59-1.11
Calcium (mg/dL)	9.13	1.07	7.90	9.80	8.8-10.3
Phosphorus (mg/dL)	5.95	1.91	4.60	7.30	2.5-5
Alkaline Phosphatase (U/L)	121.33	79.00	35.00	190.00	30-150
Alanine Aminotransferase (U/L)	25.15	3.04	23.00	27.30	0-40

Table 15. Values of blood biochemistry parameters of naturally infected dogs (n=2) with *A. platys* at the first visit

	Mean	Std. Deviation	Minimum	Maximum	Reference Range
Blood Urea Nitrogen (mg/dL)	32.80	16.12	21.40	44.20	10.0-20.0
Creatinine (mg/dL)	1.35	0.92	0.70	2.00	0.6-1.2
Total Protein ((gm/dL)	6.15	0.64	5.70	6.60	5.0-8.0
Albumin (gm/dL)	2.90	1.84	1.60	4.20	2.8-4
Globulin (gm/dL)	3.25	2.47	1.50	5.00	2,7-4.4
Albumin/Globulin Ratio	1.55	1.77	0.30	2.80	0.59-1.11
Calcium (mg/dL)	9.75	0.07	9.70	9.80	8.8-10.3
Phosphorus (mg/dL)	5.95	1.91	4.60	7.30	2.5-5
Alkaline Phosphatase (U/L)	87.00	73.54	35.00	139.00	30-150
Alanine Aminotransferase (U/L)	25.15	3.04	23.00	27.30	0-40

All the *Ehrlichia canis* dogs that were treated by Imidocarb dipropionate or a combination of Imidocarb dipropionate and doxycycline recovered. Those that were treated with doxycycline 60% recovered while 40% did not clinically recover from the infection (Figure 20). There was 100% recovery for the *Ehrlichia chaffeensis* and *Anaplasma platys* dogs.
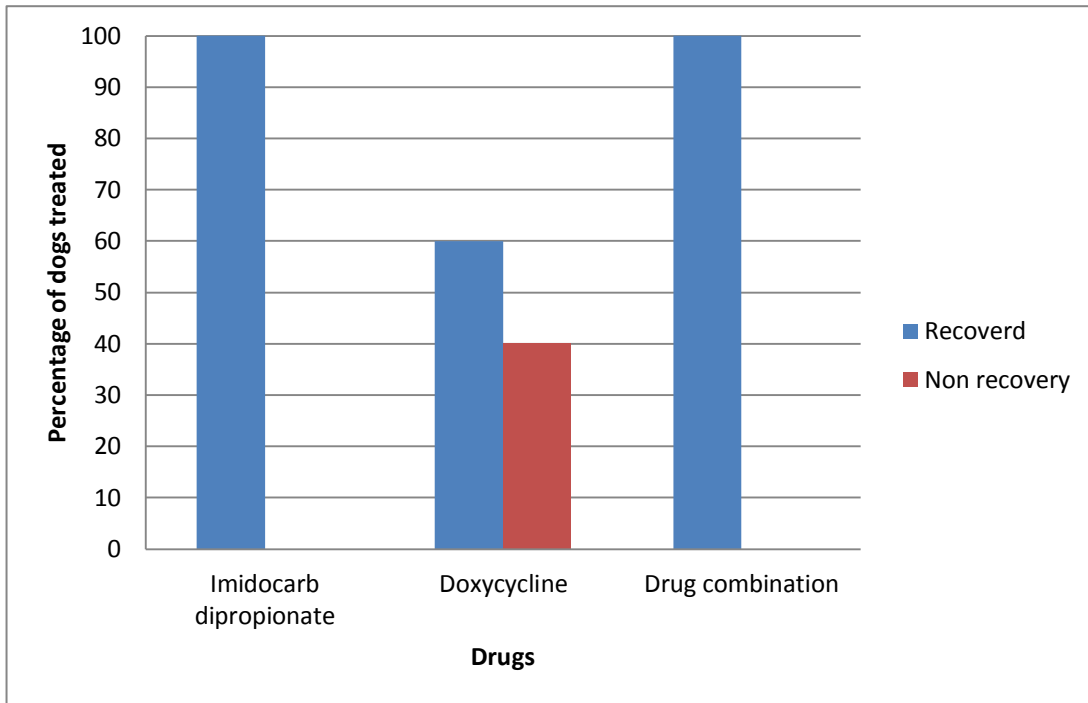
Figure 20. Percentage of dogs infected with E. canis response to treatment by Imidocarb dipropionate, doxycycline, and a combination of Imidocarb and doxycycline.

5.4. Discussion

Polymerase Chain Reaction is a very sensitive diagnostic test for *Ehrlichia* and *Anaplasma* infection that has been widely used in diagnosis even before antibodies are detectable (Wen *et al*. 1997). In this study, nested PCR which has been shown to have a ten-fold increase in sensitivity (Chang and Pan, 1996), yielded DNA fragments with *Ehrlichia* and *Anaplasma* species primers. The second PCR reactions with primers specific for *Ehrlichia canis*, *Ehrlichia chaffeensis* and *Anaplasma platys* yielded PCR products, indicating that these species were present in the blood of the dogs sampled. The specificity of the HE1 and HE3 primers used in the present study for amplification of *Ehrlichia chaffeensis* has previously been established (Anderson *et al*. 1992; Dawson *et al*. 1996). Moreover, the specificity of these primers among three closely related *Ehrlichia* species has been demonstrated by Murphy *et al*. (1998) in their study in dogs and ticks in Oklahoma. Therefore, the finding of the positive fragments by nested PCR for the respective species establishes the presence of *Ehrlichia canis*, *Ehrlichia chaffeensis* and *Anaplasma platys* in Kenya, previously not published in scientific literature.

Ehrlichia canis which infects monocytes and is the causative agent of the classical canine ehrlichiosis was identified in 42.5% of the positive results in this study confirming this species as the most prevalent cause of canine ehrlichiosis in Kenya. This study's finding is comparable to that of Romero *et al*. (2011) where nested PCR revealed 47.7% positive and Unver *et al*. (2001a) where PCR analysis using *Ehrlichia canis* species specific primers revealed that 17 of the 55 dog blood samples (31%) were positive. Although,

Ehrlichia canis was thought to be strictly a canine parasite (Anderson *et al*. 1992) isolation, antigenic and genetic characterization of an *Ehrlichia canis* strain isolated from a human in Venezuela, demonstrated human infection with *Ehrlichia canis* (Perez *et al*. 1996). This therefore, indicates the need for surveillance of humans for infection by *Ehrlichia canis* as it is potentially zoonotic.

Ehrlichia chaffeensis which was originally isolated and characterized as a cause of human disease has been isolated from dogs, where it was found to cause severe manifestation (Breitschwerdt *et al*. 1998a; Dawson *et al*. 1996); it was identified in 5.3% of the positive samples. The present study's detection of the 16S rRNA gene of *Ehrlichia chaffeensis* by PCR in naturally infected dogs is in agreement with the report of Dawson *et al*. (1996) in dogs in southern Virginia. The identification of this species is important given that Breitschwerdt *et al*. (1998a) reported that dogs naturally infected with *Ehrlichia chaffeensis* have severe disease manifestations that are clinically indistinguishable from disease manifestations of *Ehrlichia canis* or *Ehrlichia ewingii*. The identification of *Ehrlichia chaffeensis* makes it imperative for veterinary practitioners to consider zoonotic infection by this species as they manage sick animals under their care.

Imidocarb dipropionate treatment resulted in improvement and resolution of most clinical signs of ehrlichial infections in all dogs that received this drug. This indicates that Imidocarb dipropionate was efficacious against *Ehrlichia canis, Ehrlichia chaffeensis* and

Anaplasma platys in this geographic region. With Doxycycline treatment, 60% of the *Ehrlichia canis* infected dogs recovered. This is in contrast to what has been reported elsewhere (Gaunt *et al*. 2010; Schaefer *et al*. 2008; Eddlestone *et al*. 2007; Breitschwerdt *et al*. 1998b) who have reported clearance of infection by this drug. It has been reported that in experimental infections by *Ehrlichia canis* and *Anaplasma platys* doxycycline was an efficacious therapy when administered for a period of four weeks (Gaunt *et al*., 2010; Schaefer *et al*., 2008; Eddlestone *et al*. 2007). Breitschwerdt *et al.* (1998b) reported elimination of *Ehrlichia canis* in experimental infection by doxycycline and concluded that the drug is effective in the treatment of acute ehrlichiosis caused by this organism. In the present study, doxycycline was administered for a period of two weeks and this may account for the reduced efficacy observed. However, non-clearance of *Ehrlichia canis* in experimentally infected dogs by doxycycline administered over 7 days has been reported (Igbal and Rikihisa, 1994b). Breitschwerdt *et al*. (1996) also noted persistence of *Ehrlichia chaffeensis* DNA for one year following treatment with doxycycline an indication that the dogs may have been more refractory to this treatment. It can therefore, be concluded that the possible causes of non-response to doxycycline treatment in the dogs with *Ehrlichia canis,* in this study, may be noncompliance by the owners in administration of the drug or the duration of drug administration may have been inadequate.

The identification of *Ehrlichia chaffeensis* and *Ehrlichia canis* in the dog population in Kenya presents a challenge to both veterinarians and public health professionals. Particular attention should be given to the possibility of these infections more so with *Ehrlichia chaffeensis* which is mainly a human pathogen. This is especially so as

scientific reports on molecular identification of the species causing infection in dogs in Kenya is scant. This study represents the first molecular evidence of the diagnosis and characterization of *Ehrlichia canis, Ehrlichia chaffeensis* and *Anaplasma platys* in dogs in Kenya.

Although, amplifications with *Anaplasma* and *Ehrlichia* species primers yielded DNA fragments, a number of samples were negative with the nested PCR reactions using species specific primers. For these negative samples, future studies need to be done to determine the DNA sequences of these particular 16S rRNA gene fragments and compare them with those of *Ehrlichia ewingii* and *Anaplasma phagocytophilum* that were not amplified in the present study.

Studies are required to determine the DNA sequences of these particular 16S rRNA gene fragments and compare them with those identified in other parts of the world.

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CHAPTER 6

General Discussion

This work has noted the German shepherd dog to be infected with Ehrlichial organisms more frequently than the other breeds. The higher numbers may be explained by the popularity of this breed as guard dogs, though data on the breed prevalence for the country were not available. A study by Van Heerden (1982) found a higher incidence, mortality rate and chronicity among German shepherd dogs infected with *Ehrlichia canis* in South Africa. This apparent higher susceptibility of the German shepherd dog to ehrlichiosis in this study has also been previously reported (Singh *et al*. 2014; Huxsoll *et al*. 1972; Van Heerden, 1982). This susceptibility may be due to a defective cell-mediated immune response within this breed of dogs (Singh *et al*. 2014). However, Liddel *et al*. (2003) did not observe any differences among dogs with or without confirmed ehrlichiosis by sex, age, breed or fertility status.

Haematological changes usually occur in animals infected with Ehrlichia and Anaplasma. In the present study, thrombocytopenia, which is considered the most common haematological abnormality in dogs either naturally or experimentally infected by a wide range of *Ehrlichia* species (Harrus *et al*. 1997b; Harrus *et al*. 1999; Neer *et al*. 2002; Poitout *et al*. 2005) was present. Similar observations have been reported elsewhere (Tsachev *et al*. 2013; Dagnone *et al*. 2003; Woody and Hoskins, 1991). It has been suggested that platelets destruction is related to an immunological response in the infected animal (Burghen *et al.* 1971)as the presence of circulating serum antibodies has been demonstrated (Waner *et al*. 1995).Though, the reduction in numbers may also be

due to other mechanisms like increased platelet consumption (Smith *et al.*, 1974; Ristic and Holland, 1993); splenic pooling in enlarged spleens, and increased platelets destruction by the spleen (Smith *et al.* 1974) and suppression of platelet production in the bone marrow, mainly in the chronic phase (Woody and Hoskins, 1991).

Previous studies have reported lymphopenia in *Anaplasma phagocytophilum* infection in dogs (Poitout *et al.* 2005) and in *Ehrlichia canis* infection (Tsachev *et al.* 2013; Pasa and Azizogly, 2003). Significant (p<0.05)increase in mature lymphocyte from 24.28±1.03 to 29.38±1.91 on initial diagnosis and 14 days post treatment was observed in this study. This increase in lymphocytes is an indication of the recovery of these dogs from the infection upon treatment. Moreover, the thrombocytopenia noted when the dogs were presented had resolved, with the thrombocyte values returning to within the normal values 14 days post treatment.

Tsachev *et al.* (2013) noted that variations in haematological profiles in *Ehrlichia canis* infected dogs maybe related to differences in the virulence of the *Ehrlichia canis* strains, antigen heterogeneity of this bacterial agent and the clinical form of the disease.

In this study, there was increase, though not significant, in the albumin levels in the dogs between the first visit and the second visit which is a pointer to the fact that the animals were recovering. Infection with *Ehrlichia* parasites is associated with Hypoalbuminemia. According to Harrus *et al.* (1996) Hypoalbuminemia is seen in all stages of canine ehrlichiosis and maybe a consequence of anorexia and associated decrease in protein uptake, blood loss, peripheral loss to oedematous inflammatory fluids as a consequence of vasculitis (Woody and Hoskin, 1991), decreased protein production due to concurrent

liver disease (Reardon and Pierce, 1981), or due to proteinuria. Studies have indicated that proteinuria may occur independently or concurrently with glomerulonephritis (Frank and Breitschwerdt, 1999; Codner and Maslin, 1992; Waddle and Littman, 1988). The current study noted inappetence in a high percentage of the dogs in both the retrospective and prospective studies which make anorexia a very likely cause of the alteration in albumin levels observed. Marked serum protein alterations in dogs naturally infected with *Ehrlichia canis* has been reported (Harrus *et al*. 1996).

A number of the dogs clinically and microscopically diagnosed with ehrlichiosis did not yield any product with the genus primers ECC and ECB, an indication that these dogs may not have been actively carrying the infection at the time of sampling. This is especially as PCR is an effective and extremely sensitive method for the detection of *Ehrlichia* and *Anaplasma* species in dog blood and tissues (Dawson *et al.* 1996; Engvall *et al*. 1996; McBride *et al*. 1996; Iqbal and Rikihisa 1994a; Iqbal *et al*. 1994). It is also possible that what were interpreted as morulae in the blood smears from these dogs were inclusion bodies due to other causes and not Ehrlichia and Anaplasma agents. Morulae need to be differentiated from other inclusions that may be present during severe bacterial infections (like Dohle bodies), inflammation, auto-immune diseases, viral infections (like canine distemper) and severe tissue destruction which might result in false positive results (Schalm, 2000).

Breitschwerdt *et al.* (1998a) reported that dogs naturally infected with *Ehrlichia chaffeensis* have severe disease manifestations that are clinically indistinguishable from disease manifestations of *Ehrlichia canis* or *Ehrlichia ewingii*. In this study, lethargy,

lymphadenopathy, congestion of mucous membranes and inappetence were consistently present in dogs confirmed by PCR to be infected with *Ehrlichia chaffeensis*. Most of these dogs were also found to have harsh lung sounds on auscultation and tender abdomen on palpation. The most consistent clinical manifestations in dogs with *Ehrlichia canis* were lymphadenopathy, congestion of mucous membranes, inappetence, panting, loose hair, lethargy, vomiting, wasting, ocular discharge and diarrhoea. The consistent clinical signs noted in the dog confirmed by PCR to have *Anaplasma platys* were lethargy, lymphadenopathy, ocular discharge, inappetence, panting, pounding heart, harsh chest, loose hair and wasting. It has been observed that it is difficult to reach a definitive diagnosis based only on clinical and haematological abnormalities (Asgarali *et al*. 2012). This is due to the fact that natural infections may present with a variety of clinical signs in different geographical regions (Asgarali *et al*. 2012). The clinical signs noted with the various species identified in this study may be taken to give the clinical case definitions of the disease. These case definitions may be useful to the veterinary practitioner in areas with inadequate resources where advanced diagnostic tools may not be available.

The detection of morulae in stained blood smears is a valuable diagnostic tool in acute disease (Mylonakis *et al*. 2003; Hildebrandt *et al*. 1973). However, it has been observed that intracellular pathogens are notoriously difficult to detect by clinical pathological investigations (Martin *et al.* 2005). Examination of blood smears for inclusions of the parasites is usually time consuming and not very rewarding as the morulae are often absent or present in very low numbers (Woody and Hoskins, 1991; Harrus *et al*. 1997c). Despite this, it is the main method used by veterinary practitioners to confirm a diagnosis of ehrlichial infection in Kenya. Rikihisa (1999) in a review on *Ehrlichia chaffeensis*

observed that diagnosis of human monocytic ehrlichiosis is still dependent on evaluation of clinical, laboratory, and epidemiologic data. This is because confirmatory diagnostic tests are seldom available to practitioners during the active phase of the disease. These observations may also be right for veterinary practitioners dealing with animals infected by *Ehrlichia* and *Anaplasma* species. Therefore, the identification of the species present in Kenya will assist in availability of epidemiologic data to assist practitioners in making a diagnosis of these infections in view of the limitations of the availability of the diagnostic services.

Although, laboratory diagnosis is mostly done by IFA test, caution must be exercised in immunocompromised patients, because they may not seroconvert (Paddock *et al*. 1997; 1993). Moreover, IFA test cannot distinguish infections with *Ehrlichia chaffeensis* from infections with *E. canis* or other related organisms and if the infection is at an early stage, the test results may be negative (Rikihisa, 1999). Therefore, this study a PCR assay, based on the 16S rRNA sequence of the Lousiana isolate of *Ehrlichia canis* that is highly specific and sensitive (McBride *et al*. 1996), was used. Several studies have shown PCR to be an effective and extremely sensitive method of detection of *Ehrlichia* species in dog blood and tissues (Chang and Pan, 1996; Dawson *et al*. 1996; Engvall *et al*.1996; McBride *et al*. 1996; Iqbal and Rikihisa, 1994a; Iqbal *et al*. 1994). Using a nested PCR which is even more sensitive (Chang and Pan, 1996), existence of *Ehrlichia canis*, *Ehrlichia chaffeensis* and *Anaplasma platys* in dogs in Nairobi, Kenya was confirmed. Scientific reports on molecular identity of ehrlichial species have not been previously published. Establishment of these infections is important since tick-borne diseases are becoming increasingly important throughout the world and monitoring of their causative

agents in the field may serve as a useful indicator of potential human exposure (Masala *et al*. 2012). Among these tick-borne infections are those caused by *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis* and *Ehrlichia ewingii* (Sirigireddy and Ganta, 2005). One of these, *Ehrlichia chaffeensis*, was identified in this study. Infection of a human by *Ehrlichia canis* has also been reported in Venezuela, South America (Perez *et al*. 1996) implicating this agent as a zoonotic organism.

This study of ehrlichial species present in dogs in Nairobi revealed several notable results. The major observation is that more than 42% of dogs with molecular evidence of current ehrlichial infection were caused by *Ehrlichia canis*. Although, this species had previously been demonstrated to be the cause of infection in Kenya by culture and serology (Alexander *et al*. 1994; Price, 1980; Kaminjolo *et al*. 1976; Nyindo *et al*.1971) no published data has reported molecular characterization of ehrlichial infections in dogs in Kenya. *Anaplasma platys* infection was detected in only 3 samples, an indication that Anaplasmosis caused by this organism may not be a significant disease in the area. However, it is important to note that only a limited number of dogs were tested. Surveillance of ehrlichiosis and anaplasmosis is required involving more animals from different areas of Kenya, in order to better understand the prevalence of these diseases in the country.

This study presents the first molecular confirmation that *Ehrlichia canis, Ehrlichia chaffeensis* and *Anaplasma platys* are present in this country. Further investigations are, however, needed in order to elucidate the presence of these organisms in humans as *Ehrlichia chaffeensis* is zoonotic and *Ehrlichia canis* is potentially zoonotic. It is also

noted that congestion of mucous membranes is an important clinical sign that is present in animals infected by Ehrlichiosis and Anaplasmosis.

Conclusions

1. Congestion of mucous membranes is an important clinical manifestation of infection by *Ehrlichia canis, Ehrlichia chaffeensis* and *Anaplasma platys* infection in dogs.

2. Lymphadenopathy is an important clinical manifestation in dogs infected with *Ehrlichia* and *Anaplasma*. As it was observed in 65.3% and 59.3% of the dogs in the prospective and retrospective studies respectively.

3. Imidocarb dipropionate is still the drug of choice in the therapeutic management of Ehrlichia and Anaplasma infections.

4. There is very low use of non injectables in the therapeutic management of ehrlichiosis and anaplasmosis.

5. Client education may be an important consideration in ensuring drug efficacy in the use of doxycycline.

6. *Ehrlichia canis*, *Ehrlichia chaffeensis* and *Anaplasma platys* are present in the dogs in our region.

Recommendations

1. Studies are required to determine the DNA sequences of the 16S rRNA gene fragments that were positive for the genera but negative for species and compare them with those identified in other parts of the world.

2. Studies need to be undertaken to find out the reason for the low client compliance in the management of ehrlichiosis and anaplasmosis in order to implement appropriate intervention.

7.0   REFERENCES

Al-Badrani B.A. (2013): Diagnostic study of ehrlichiosis in cattle of Mosul-Iraq. Basrah Journal of Veterinary Research **12**: 87-97.

Alexander K.A., Kat P.W., Wayne R.K. and **Fuller T.K**. **(1994):** Serologic survey of selected canine pathogens among free-ranging jackals in Kenya. Journal for Wildlife Diseases.**30:** 486-391.

Alexandre N., Santos A.S., Nuncio M.S., de Sousa R., Boinas F. and **Bacellar F. (2009):** Detection of *Ehrlichia canis* by polymerase chain reaction in dogs from Portugal. The Veterinary Journal.**181**: 343-344.

Anderson B.E., Green J.E, Jones D.C. and **Dawson J.E**. **(1992):** *Ehrlichia ewingii* sp. nov., the etiologic agent of canine granulocytic ehrlichiosis. International Journal of Systematic and Evolutionary Microbiology.**42**: 299-302.

Anderson B.E., Dawson J.E., Jones D.C. and **Wilson K.H. (1991):** *Ehrlichia chaffeensis*, a new species associated with human ehrlichiosis. Journal of Clinical Microbiology.**29**: 2838-2842.

Anziani A.S., Ewing S.A. and **Barker R.W. (1990):** Experimental transmission of a granulocytic form of the tribe Ehrlichieae by *Dermacentor variabilis* and *Amblyomma americanum* to dogs. American Journal of Veterinary Research.**51**: 929-931.

Asgarali Z., Pargass I., Adam J., Mutani A. and **Ezeokoli C. (2012):** Haematological parameters in stray dogs seropositive and seronegative to Ehrlichia canis in North Trinidad. Ticks and Tick Borne Diseases. **3**: 207-211.

Bakken J.S., Krueth J.K., Lund T, **Malkovitch D., Asanovich, K**. and **Dumler, J.S.(1996):** Exposure to deer blood may be a cause of human granulocytic ehrlichiosis. Clinical Infectious Diseases.**23**:198.

Baumgarten B.U., Rollinghoff M. and **Bogdan C. (1999**): Prevalence of *Borrelia burgdorferi* and granulocytic and monocytic ehrlichiae in *Ixodes ricinus* ticks from Southern Germany. Journal of Clinical Microbiology.**37**: 3448-3451.

Bexfield N.H., Villiers E.J. and **Herrtage M.E. (2005**): Immune-mediated haemolytic anaemia and thrombocytopenia associated with *Anaplasma phagocytophilum* in a dog. Journal of Small Animal Practice.**46**: 543-548.

Bostros B.A., Elmolla M.S., Salib A.W., Calamaio C.A, Dasch G.A. and **Arthur R.R. (1995**): Canine ehrlichiosis in Egypt: seroepidemiological survey. Onderstepoort Journal of Veterinary Research.**62**: 41-43.

Bjoerdroff A., Svendenius L., Owens J.H. and **Massung R.F. (1999**): Feline granulocytic ehrlichiosis- a report of a new clinical entity and characterization of the infectious agent. Journal of Small Animal Practice.**40**:20-24.

Buller R.S., Arens M., Hmiel S.P., Paddock C.D., Sumner J.W., Rikihisa Y., Unver A., Gaudreault-Keener M., Manian F.A., Liddell A.M., Schmulweitz N. and

Storch G.A. (1999): *Ehrlichia ewingii*, a newly recognized agent of human ehrlichiosis. New England Journal of Medicine.**341**: 148-155.

Buhles W.C., Huxsoll D.L. and **Hildebrandt P.K. (1975):** Tropical canine pancytopenia: role of aplastic anemia in the pathogenesis of severe disease. Journal Comparative Patholology.**85**: 511–521.

Buoro I.B.J., Atwell R.B. and **Kiptoon J. (1986**): Feline anemia associated with ehrlichia- like bodies in three domestic shorthaired cats. Veterinary Record.**125**: 434-436.

Burghen G.A**., Beisel W.R., Walker J.S., Nims R.M., Huxsoll D.L**. and **Hildebrandt P.K. (1971**): Development of hyperglobulinemia in tropical canine pancytopenia. American Journal of Veterinary Research.32: 749-756.

Breitschwerdt E.C. (2000): The Rickettsioses In: Textbook of Veterinary Internal Medicine. 5th Edition. Editors S.J. Ettinger and Feldman. WB Saunders, Philadelphia. Pp 400-408.

Breitschwerdt E.B., Hegarty B.C. and **Hancook S.I. (1998a):** Sequential evaluation of dogs naturally infected with *Ehrlichia canis, Ehrlichia chaffeensis, Ehrlichia equi, Ehrlichia ewingii*, or *Bartonella vinsonii*. Journal of Clinical Microbiology **36**:2645-2651.

Breitschwerdt E.B., Hegarty B.C. and **Hancook S.I. (1998b):** Doxycycline hyclate treatment of experimental canine ehrlichiosis followed by challenge inoculation with two *Ehrlichia canis* strains. Journal of Clinical Microbiology **42**: 362-368.

Brouqui P., Davoust B., Haddad S., Vidor V. and **Raoult D. (1991):** Serological evaluation of *Ehrlichia canis* infections in military dogs in Africa and Reunion Island. Veterinary Microbiology.**26**: 103-105.

Brouqui P. and **Dumler J.S. (1997):** The immune response to *Ehrlichia chaffeensis*. In Anderson, B., Friedman, H., Bendinelli, M. (Eds.), Rickettsial infection and immunity. Plenum Press, New York (London), pp. 163-172.

Brown G.K., Martin A.R., Robert T.K. and **Aitken R.J. (2001**): Detection of *Ehrlichia platys* in dogs in Australia. Australian Veterinary Journal.**79**: 554-558.

Carlyon J.A. and **Fikrig E. (2003):** Invasion and survival strategies of Anaplasma phagocytophilum. Cellular Microbiology.**5**: 743-754.

Castro de M.B., Mchado R.Z, Aquino de L.P.C.T., Alessi A.C. and **Costa M.T. (2004):** Experimental acute canine monocytic ehrlichiosis: Clinicopathological findings. Veterinary Parasitology.**119**:73-86.

Chang W.L. and **Pan M.J. (1996**): Specific amplification of *Ehrlichia platys* DNA from blood specimens by two-step PCR. Journal of Clinical Microbiology.**34**: 3142-3146.

Clark A.M., Hopkins G.F. and **MacLean I.A. (1996):** Tick-borne fever in dogs. Veterinary Record.**139**: 268.

Codner E.C. and **Maslin W.R. (1992)**: Investigation of renal protein loss in dogs with acute experimentally induced *Ehrlichia canis* infection. American Journal of Veterinary research.**53**: 294-299.

Cohn L.A. (2003): Ehrlichiosis and related infections. Veterinary Clinics Small Animal.**33**:863-884.

Coles E.H. (1986): Veterinary Clinical Pathology. 4th ed. WB Saunders Company London, UK.46-47.

Danks W.B.C. (1937): Rickettsia infection of the dog. Annual Report Veterinary Department. Kenya 64-66.

Dagnone A., De Morais H., Vidotto M, Jojima F. and **Vidotto O. (2003)**: Ehrlichiosis in anemic thrombocytopenic or tick-infested dogs from hospital population in south Brazil. Veterinary Parasitology.**117**: 285-290.

Dawson J.E., Stallknecht E., Howerth E.W., Warner C., Biggie K., Davidson W.R., Lockhart J.M., Nettles V.F., Olson J.G. and **Childs J.E. (1994):** Susceptibility of white-tailed deer (*Odocoileus virginianus*) to infection with *Ehrlichia chaffeensis* the etiologic agent of human ehrlichiosis. Journal of Clinical Microbiology. 32: 2725-2728.

Dawson J.E., Biggie K., Warner C.K., Cookson K., Jenkins S., Levine J.F. and **Olson J.G. (1996**): Polymerase chain reaction evidence of *Ehrlichia chaffeensis*, etiologic agent of human ehrlichiosis, in dogs from southwest Virginia. American Journal of Veterinary Research.**57**:1175-1179.

Diez R.A., Ramos J.A., Lopez R.M.A. and **Gil E.B. (1988**): Boutonneuse fever transmitted by conjunctival inoculation. Klinische Wochenschrift. **66**:1212-1213.

Dumler J.S., Asanovich K.M., Bakken J.S., Richter P., Kimsey R. and **Madigan J.E. (1995):** Serologic cross-reactions among *Ehrlichia equi, Ehrlichia phagocytophila* and human granulocytic ehrlichia. Journal of Clinical Microbiology.**33**:1098-1103.

Dumler J.S., Barbet A.F., Bekker CPJ, Dasch GA, Palmer GH, Rikihisa Y. and **Rurangirwa F. (2001):** Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales; unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia*, and *Ehrlichia* with *Neorickettsia*; description of five new species combinations: and designation of *Ehrlichia equi* and HGE agent as subjective synonyms of *Ehrlichia phagocytophila*. International Journal of Systematic and Evolutionary Microbiology.**51**:2145-2165.

Eddlestone S.M., Diniz P.P.V.P., Neer T.M., Corstvet R., Cho D., Hosgood G., Hergarty B. and **Breitschwerdt E.B. (2007):** Doxycycline clearance of

experimentally induced chronic Ehrlichia canis infection in dogs. Journal of Veterinary Internal Medicine.**21**: 1237-1242.

Engvall E.O., Pettersson B., Persson M. Artursson K. and **Johansson K.E. (1996):** A 16S rRNA –based PCR assay for detection and identification of granulocytic Ehrlichia species in dogs, horses, and cattle. Journal of Clinical Microbiology.**34**: 2170-2174.

Engvall A., Hedhammer A. and **Bjoersdorff I. (1997):** Clinical features and serology of 14 dogs affected by granulocytic ehrlichiosis in Sweden. Veterinary Record.**140**: 222-226.

Ewing S.A., Robertson W.R., Buckner R.G. and **Hayat C.S. (1971):** A new strain of *ehrlichia canis*. Journal American Veterinary Association.**159**: 1771-1774.

Ewing S.A., Dawson J.E., Kocan A.A., Barker R.W., Warner C.K., Panciera R.J., Fox J.C., Kocan K.M. and **Blouin E.F. (1995):** Experimental transmission of *Ehrlichia chaffeensis* (Rickettsiales: Ehrlichieae) among white-tailed deer by *Amblyomma americanum* (*Acari; Ixodidae*). Journal of Medical Entomology.**32**: 368-374.

Farias Rotondano T.E., de Almeida A.M.P., Lustosa E.M.C., de Azevedo S.S., de Andrade P.P. and **de Melo M.A. (2012):** An assessment of whole blood and fractions by nested PCR as a DNA source for diagnosing canine ehrlichiosis and anaplasmosis. The Scientific World Journal. Article ID 605743, 6 pages doi: 10.1100/2012/605743.

Frank J.R. and **Breitschwerdt E.B. (1999):** A retrospective study of ehrlichiosis in 62 dogs from North Carolina and Virginia. Journal of Veterinary Internal Medicine.**13**:194-201.

Gaunt S.D., Beall M.J., Stillman B.A., Lorentzen L., Diniz P.P.V.P., Chandrashekar E.B. and **Breitschwerdt E.B. (2010):** Experimental infection and co-infection of dogs with *Anaplasma platys* and *Ehrlichia canis*: hematologic, serologic and molecular findings. Parasites and Vectors.**3**: 33-43.

Goldman E.E., Breitschwerdt E.B., Grindem C.B., Hegarty B.C., Walls J.J and **Dumler J.S. (1998):** Granulocytic ehrlichiosis in dogs from North Carolina and Virginia. Journal of Veterinary Internal Medicine.**12:** 61-70.

Goodman R.A., Hawkins E.C., Olby N.J., Grindem C.B., Hegarty B. and **Breitschwerdt E.B. (2003):** Molecular identification of *Ehrlichia ewingii* infection in dogs: 15 cases (1997-2001). Journal of American Veterinary Medical Assosiation.**222**: 1102-1107.

Gould D.J., Murphy K., Rudorf H. and **Crispin S.M. (2000):** Canine monocytic ehrlichiosis presenting as acute blindness 36 months after importation in the UK. Journal of Small Practice.**41**:263-265.

Granick J.K., Armstrong P.J. and **Blender J.B.** (**2009):** *Anaplasma phagocytophilum* infection in dogs: 34 cases (2000-2007). Journal of American Veterinary Medical Association.**234**: 1559-1565.

Greig A., MacLeod S. and **Allison J. (1977):** Tick-borne fever in association with mucosal disease and cobalt deficiency in calves. Veterinary Record.**100**: 562–564.

Greig B., Asanovich K.M., Amstrong P.J. and **Dumler J.S. (1996):** Geographic, clinical, serologic and molecular evidence of granulocytic ehrlichiosis, a likely zoonotic disease, in Minnesota and Wisconsin dogs. Journal Clinical Microbiology.**34**: 44-48.

Grindem C.B., Breitschwerdt E.B., Corbett W.T. and **Jans H.E. (2002):** Epidemiologic survey of thrombocytopenia in dogs: a report on 987 cases. Veterinary Clinical Pathology.**20**: 38-43.

Goldman E.E., Breitschwerdt E.B., Grindem C.B., Hegarty B.C., Walls J.J. and **Dumler J.S. (1998**): Granulocytic Ehrlichiosis in Dogs from North Carolina and Virginia. Journal of Veterinary Internal Medicine.**12**:.61-70.

Groves M.G., Dennis G.L., Amyx H.L. and **Huxsoll D.L. (1975):** Transmission of *Ehrlichia canis* to dogs by ticks (*Rhipicephalus sanguineus*). American Journal of Veterinary Research.**36**: 937-940.

Harrus S., Waner T., Strauss-ayali D., Bark H., Jongeja F., Hecht G. and **Baneth G. (2001**): Dynamics of IgG 1 and IgG 2 subclass response indogs naturally and experimentally infected with *Ehrlichia canis*. Veterinary Parasitology.**99**: 63-71.

Harrus S., Kass P.H., Klement E. and **Waner T. (1997a):** Canine monoctyic ehrlichiosis: a retrospective study of 100 cases, and an epidemiological

investigation of prognostic indicators for the disease. Veterinary Record.**141**: 360-363.

Harrus S., Waner T. and **Bark T. (1997b)**: Canine monocytic ehrlichiosis- an update. Compendium of Containing Education for Practicing Veterinarian.**19**: 431-444.

Harrus S., Aroch I., Lavy E. and **Bark H. (1997c**): Clinical manifestation of canine cyclic thrombocytopenia. Veterinary Record.**141**:247-250.

Harrus S., Waner T., Avidar Y., Bogin E., Peh H.C. and **Bark H. (1996):** Serum protein alterations in canine ehrlichiosis. Veterinary Parasitology.**66**: 241-249.

Harrus S., Waner T. Aizenberg I. and **Bark T. (1998):** Therapeutics effect of Doxycycline in experimental sub clinical canine monocytic ehrlichiosis: evaluation of a 6 week course. Journal of Clinical Microbiology.**36**: 2140-2142.

Harrus S., Waner T., Bark T., Jongeja F. and **Cornelisen A.W.C.A. (1999**): Recently advances in determining the pathogenesis of canine monocytic ehrlichiosis. Journal of Clinical Microbiology.**37**: 2745-2749.

Harvey J.W., Simpson C.F. and **Gaskin J.M. (1978):** Cyclic thrombocytopenia induced by a Rickettsia-like agent in dogs. Journal of infectious Disease. 137: 182-188.

Hawkins J.A., Love J.N. and **Hidalgo R.J. (1982):** .Mechanical transmission of anaplasmosis by tabanids (Diptera: Tabanidae). American Journal of Veterinary Research. **43**: 732-734.

Hildebrandt A., Huxsoll D.L., Walker J.S., Nims R.M., Taylor R. and **Andrews M. (1973):** Pathology of canine ehrlichiosis (Tropical canine pancytopenia). American Journal of Veterinary Research.**34**: 1309-1320.

Hildebrandt A., Schmidt K.H., Fingerle V., Wilske B.and, **Straube E. (2002):** Prevalence of granulocytic Ehrlichiae in *Ixodes ricinus* ticks in Middle Germany (Thuringia) detected by PCR and sequencing of a 16S ribosomal DNA fragment. FEMS Microbiology Letters.**211**: 225–230.

Horowitz H.W, Kilchevsky E, Haber S, Rosenfeld M.A., Kranwinkel R., James E.K., Wong S.J., Chu F., Liveris D. and **Schwartz I. (1998):** Perinatal transmission of the agent of human granulocytic ehrlichiosis. New England Journal of Medicine.**339**:375-378.

Huxsoll D.L., Amyx H.L., Hemelt I.E., Hildebrandt P.K., Nims R.M. and **Gochenour W.S. (1972):** Laboratory studies of tropical canine pancytopenia. Experimental Parasitology.**31**: 53-59.

Inokuma H., Ohno K., Onishi T., Raoult D. and **Brouqui P. (2001):** Detection of Ehrlichial infection by PCR in dogs from Yamaguchi and Okinawa prefectures, Japan. Journal Veterinary Medical Science.**63**:815-817.

Iqbal Z. and **Rikihisa Y. (1994):** Application of the polymerase chain reaction for the detection of Ehrlichia canis in tissues of dogs. Veterinary Microbiology.**42**: 281-287.

Iqbal Z. and **Rikihisa Y. (1994b):** Reisolation of *Ehrlichia canis* from blood and tissues of dogs after doxycycline treatment. Journal of Clinical Microbiology.**32**: 1644-1649.

Iqbal Z., Chaichanasiriwithaya W. and **Rikihisa Y. (1994):** Comparison of PCR with other tests for early diagnosis of canine ehrlichiosis. Journal Clinical Microbiology.**32**: 1658-1662.

Ismail N, Bloch KC and **McBride JW.(2010):** Human ehrlichiosis and anaplasmosis. Clinical Laboratory Medicine.**30**: 261-292.

Jerrels T.R. (1997): Immunity to Rickettsiae (redux). In: Anderson, B., Freidman, H., Bendinelli, M. (Eds.), Rickettsial infection and immunity. Plenum Press, New York (London).pp. 15-28.

Johnson E.M., Ewing S.A., Barker R.W., Fox J.C., Crow D.W.and **Kocan K.M. (1998):** Experimental transmission of *Ehrlichia canis* (Rickettsiales: Ehrlichieae)by*Dermacentor variabilis* (Acari: Ixodidae). Veterinary Parasitology.**74**: 277-288.

Kakoma T., Hansen R.D., Anderson B.E., Hanley T.A., Sims K.G., Lin L., Bellamy C., Long M.T. and **Baek B.K. (1994):** cultural, molecular and immunological characterization of the etiological agent for a typical canine ehrlichiosis. Journal Clinical Micribiology.**32**:170-175.

Kaminjolo J.S., Nyindo M.B.A., Sayer P.D., Rurangirwa F., Johnson L.W., Hird, S.F., Rosenbaum E., Maxie L.L.S. and **Ogaa J.S. (1976):** Identification of *Ehrlichia canis* in East Africa. Veterinary Record.**99**:434-435.

Kaur P., Deshmukh S., Singh B., Bansal B.K., Randhawa C.S. and **Singla L.D. (2011)**: Para-clinico-pathological observations of insidious incidence of canine hepatozoonosis from a mongrel dog: a case report. Journal of Parasitology and disease.**36**: 135-138.

Kenyon R.H., Kishimoto R.A. and **Hall W.C. (1979):** Exposure of guinea pigs to *Rickettsia rickettsii* by aerosol, nasal, conjunctival, gastric and subcutaneous routes and protection afforded by an experimental vaccine. Infection and Immunity.**25**: 580-582.

Kelly P.J., Eoghain G.N. and **Raoult D. (2004):** Antibodies reactive with *Bartonella henselae* and *Ehrlichia canis* in dogs from communal lands of Zimbabwe. Journal of South African Veterinary Association.**75**: 116-120.

Kim C., Kim M., Park M., Park J. and **Chae J. (2003):** Identification of *Ehrlichia chaffeensis, Anaplasma phagocytophilum* and *A. bovis* in *Haemaphysalis longicornis* and *Ixodes persulcatus* ticks from Korea. Vector-Borne and Zoonotic Diseases.**3**: 17-26.

Kordik S.K., Breitschwerdt E.B., Hegarty B.C., Southwill K.L., Colitz C.M., Hancock S.I., Bradley J.M., Rumbough R., McPherson J.T. and

MacCormack J.N. (1999): Co-infection with multiple tick pathogens in a walker Hound kennel in North Carolina. Journal of Clinical Microbilogy.**37**:2631-2638.

Kramer V.L., Randolph M.P., Hui L.T., Irwin W.E., Gutierrez A.G. and **Vugia D.J. (1999):** Detection of the agents of human ehrlichiosis in ixodid ticks from California. American Journal of Tropical Medicine and Hygiene.**60**: 62-65.

Kuhen N.F. and **Gaunt S.D. (1985):** Clinical and hematologic findings in canine ehrlichiosis. Journal of American Veterinary Medical Association.**186**:355-358.

Labruna M.B, McBride J.W., Camargo L.M.A., Aguiar D. M, Yabsley M.J., Davidson W.R., Stromdahl E.Y., Williamson P.C, Stich R.W.,Long S.W., Camargo E.P. and **Walker D.H. (2007**): A preliminary investigation of Ehrlichia species in ticks, humans, dogs, and capybaras from Brazil. Veterinary Parasitology.**143**: 189–195.

La Scola B. and **Roult D. (1999):** Serologic diagnosis of rickettsiosis. In: Raoult, D., Brouqui, P. (Eds.), Rickettsiae and Rickettsial Diseases at the Turn of the Third Millenium. Elisevier, Paris, pp. 320-329.

Leiby D.A. and **Gill J.E. (2004):** Transfusion-transmitted tickborne infections: a cornucopia of threats. Transfusion Medicine Reviews.**18**: 293-306.

Liddell A.M., Stockham S.L., Scott M.A., Sumner J.W., Paddock C.D., Gaudreault-Keener M., Arens M.Q. and Storch G.A. (2003): Predominance of *Ehrlichia ewingii* in Missouri Dogs. Journal of Clinical Microbiology, **41**: 4617–4622.

Loverin S.L., Pierce K.R. and **Adams L.G. (1980):** Serum complement and platelet adhesiveness in acute canine ehrlichiosis. American Journal of Veterinary Research.**41**: 1266–1271.

Martin A.R., Brown G.K., Dunstan R.H. and **Roberts T.K. (2005):** *Anaplasma platys*: an improved PCR for its detection in dogs. Experimental Parasitlogy.**109**: 176-180.

Masala G., Chisu V., FoxiC., Socolovschi C., Raoult D. and **Parola P. (2012):** First detection of *Ehrlichia canis* in Rhipicephalus bursa ticks in Sardinia, Italy. Ticks and Tick-borne Diseases.**3**: 396-397.

Mazepa A.W., Kidd L.B., Young K.M. and **Trepanier L.A. (2010):** Clinical presentation of 26 *Anaplasma phagocytophilum* seropositive dogs residing in an endemic area. Journal of American Animal Hospital Association.**46**: 406-412.

McBride J.W., Corstvet R.E., Gaunt S.D., Chinsangaram J., Akita G.Y. and **Osburn B.I. (1996):** PCR detection of acute *Ehrlichia canis* infection in dogs. Journal Of veterinary Diagnosis and Investigation.**8**:441-447.

Murphy G.L., Ewing S.A., Whitworth L.C., Fox J.C. and **Kocan A.A. (1998):** A molecular and serological survey of *Ehrlichia* canis, *E. chaffeensis*, and *E. ewingii* in dogs and ticks from Oklahoma. Veterinary Parasitology.**79**:325-339.

Murray M. (1967): The pathology from some diseases found in wild animals in East Africa. East Africa Wildlife Journal.**5**:37-45.

Murray M. (1968): A survey of diseases found in dogs in Kenya. Bulletin of Epizootic Diseases of Africa.**16**:121-127.

Mylonakis M.E., Saridomichelakis M.N.; Lazaridis V.; Leontides L.S, Kostoulas P. and **Koutinas A.F. (2008)**: A retrospective study of 61 cases of spontaneous canine epistaxis (1998-2001). Journal of Small Animal Practice. **49**: 191-196.

Mylonakis M.E., Siarkou I., Leontides L., Hatzopoulou E.B, Kontos V.I. and **Koutinas A.F. (2009)**: Evaluation of a serum-based PCR assay for the diagnosis of canine monocytic ehrlichiosis. Veterinary Microbiology.**138**:390-393.

Mylonakis M.E., Koutinas A.F, Billinis C., Leontides L.S., Kontos V., Papadopoulos O., Rallis T. And **Fytianou A**. (**2003**): Evaluation of cytology in the diagnosis of acute canine monocytic ehrlichiosis (*Ehrlichia canis*): A comparison between five methods. Veterinary Microbiology.**91**: 197-204.

Ndip L.M., Ndip R.N., Esemu S.N., Dickmu V.L., Fokam E.B., Walker D.H. and **McBride J.W. (2005):** Ehrlichial infection in Cameroonian canines by *Ehrlichia canis* and *Ehrlichia ewingii*. Veterinary Microbiogy.**111**: 59-66.

Neer T.M. (1995): Ehrlichiosis update. Proceedings of the 13th Annual Congress of the American College of Veterinary Internal Medicine. San Diego, California, USA. Proceedings pp 822-826.

Neer T.M. (1998): Ehrlichiosis: canine monocytic and granulocytic ehrlichiosis. In Greene GE, Editor. Infectious diseases of the dog and cat.2nd Edition. Philadelphia: WB Saunders Co. pp 19-149.

Neer T.M., Breitschwerdt E.B., Greene R.T. and **Lapin M.R. (2002):** Consensus statement on ehrlichial disease of small animals from the Infectious Disease Study Group of the ACVIM. Journal of Veterinary Internal Medicine.**16**: 309-315.

Neer T.M. and **Harrus S. (2006):** Canine monocytotropic ehrlichiosis and neorickettsiosis (*E. canis, E. chaffeensis, E. ruminantium, N. sennetsu and N. risticii* infection). In: Greene C.E. (Ed.), Infectious diseases of the dog and cat. Saunders Elsevier, St. Louis, USA, pp 203-217.

Nyindo M.B.A., Huxsoll D.L., Ristic M., Kakoma I, Brown J.L., Carson C.A. and **Stephenson E.H. (1980):** Cell mediated response of German Shepherd dogs and Beagles to experimental infection with *Ehrlichia canis*. American Journal of Veterinary Research.**41**: 250-254.

Nyindo M.B.A., Ristic M., Huxsoll D.L. and **Smith A.R. (1971):** Tropical canine pancytopenia: *in vtro* cultivation of the causative agent-*Ehrlichia canis*. American Journal of Veterinary Research.**32**:1651-1658.

Oster C.N., Burke D.S., Kenyon R.H., Ascher M.S, Harber P. and Pedersen C.E. (1977): Laboratory acquired Rocky Mountain spotted fever. The hazard of aerosol transmission. New England Journal of Medicine.**297**: 859-863.

Paddock C.D., Surchard D.P., Grumbach K.L., Hadley W.K., Kerschman R.L., Abbey N.W., Dawson J.E., Anderson B.E., Sims K.G., Dumler J.S. and **Herndier B.G. (1993):** Brief report: fatal seronegative ehrlichiosis in a patient with HIV infection. The New England Journal of Medicine.**329**: 1164-1167.

Paddock C.D., Sumner J.W., Shore M., Bartley D.C., Elie D.C., Mcquade J.G., Martin C.R., Goldsmith C.S. and **Childs J.E. (1997**): Isolation and characterization of *Ehrlichia chaffeensis* strains from patients with fatal ehrlichiosis. Journal of Clinical Microbiology.**35:** 2496-2502.

Park J. and **Rikihisa Y. (1991):** Inhibition of *Ehrlichia risticii* infection in murine peritoneal macrophages by gamma interferon, a calcium ionophore, and concanavalin A. Infection and Immunity.**59**: 3418-3423.

Pasa S. And **Azizogly A. (2003):** Clinical and some haematological findings in dogs with ehrlichiosis: 4 cases. Indian Veterinary Journal.**80**: 33-35.

Perez M., Rikihisa Y. and **Wen B. (1996):** *Ehrlichia canis* agent isolated from a man in Venezuela: antigenic and genetic characterization. Journal Clinical Microbiology.**34**:2133-2139.

Pretorius A.M. and **Kelly P.J. (1998):** Serological survey for antibodies reactive with *Ehrlichia canis* and *Ehrlichia chaffeensis* in dogs from Bloemfontein area, South Africa. Journal of South African Veterinary Assocition.**69**:126-128.

Pritt B.S, **Sloan L.M.**, **Johnson D.K.**, **Munderloh U.G.**, **Paskewitz S.M.**, **McElroy K.M.**, **McFadden J.D. Binnicker M.J.**, **Neitzel D.F.**, **Liu G.**, **Nicholson W.L.**, **Nelson C.M.**, **Franson J.J.**, **Martin S.A.**, **Cunningham S.A.**, **Steward C.R.**, **Bogumill K.**, **Bjorgaard M.E.**, **Davis J.P.**, **McQuiston J.H.**, **Warshauer D.M.**, **Wilhelm M.P.**, **Patel R., Trivedi V.A.**, and **Eremeeva M.E. (2011):** Emergence of a new pathogenic Ehrlichia species, Wisconsin and Minnesota, 2009. North England Journal of medicine. **365**: 422-429.

Poitout F.M, Shinozaki J.K., Stockwell P.J., Holland C.J. and **Shukla S.K. (2005):** Genetic variants of *Anaplasma phagocytophilum* infecting dogs in Western Washington State. Journal Clinical Microbilogy.**43**:796-801.

Price J.E. (1980). Clinical and Haematological responses of domestic dogs and free living jackals (*Canis mesomelas*) to *Ehrlichia canis* infection. PhD. Thesis. University of Nairobi.

Purnell R E, Young E.R., Brocklesby D.W. and **Hendry D.J. (1977):** The haematology of experimentally-induced *B. divergens* and *E. phagocytophila* infections in splenectomised calves. Veterinary Record.**100**: 4–6.

Ravyn M.D., Lamb L.J, Jemmerson R, Goodman J.L and **Johnson RC. (1999):** Characterization of monoclonal antibodies to an immunodominant protein of the etiologic agent of human granulocytic ehrlichiosis. American Journal of Tropical Medicine and Hygiene.**61**: 171-176.

Reardon M.J. and **Pierce K.R. (1981):** Acute experimental canine ehrlichiosis. Sequential reaction of the hemic and lymphoreticular systems. Veterinary Pathology.**18**: 48-61.

Reddy G.R., Susana C.R., Barbet A.F., Mahan S.M., Barrage M.J. and **Allen man A.R. (1998):** Molecular characterization of a 28 kva surface antigen gene family of the tribe Ehrlichia. Biochemical and Biophysical Research Communication.**247**:636-643.

Rikihisa Y. (1999): Clinical and biological aspects of infection caused by *Ehrlichia chaffeensis*. Microbes and Infection.**1**: 367-376.

Rikihisa Y. (1991): The tribe Ehrlichieae and ehrlichial diseases. Clinical Microbiology Review.**4**: 286-308.

Rikihisa Y., Ewing S.A., Fox J.C., Siregar A.G., Pasaribu F.H. and **Malole M.B. (1992):** Analyses of *Ehrlichia canis* and a canine granulocytic ehrlichia infection. Journal of Clinical Microbiology.**30**: 143-148.

Ristic M. and **Holland C.J. (1993):** Canine ehrlichiosis. In: Woldehiwet, Z. Ristic M (Eds.), Rickettsial and Chlamydial diseases of domestic animals. Pergamon Press, Oxford, pp 169-186.

Ristic M., Huxsoll D.L., Weisiger R.M., Hildebrandt P.K. and **Nyindo M.B.A. (1972):** Serological diagnosis of tropical canine pancytopenia by indirect immunofluorescence. Infection and Immunity.**6**:226-231.

Romero L.E., Meneses A.I., Salazar L., Jimenez M., Romero J.J., Agniar D.M., Labruna M.B. and **Dolz G. (2011**): First isolation and molecular characterization of *Ehrlichia canis* in Costa Rica, Central America. Research in Veterinary Science.**91**: 95-97.

Santos F., Coppede J.S., Pereira A.L.A., **Oliveira L.P., Roberto P.G., Benedetti R.B.R., Zucoloto L.B., Lucas L., Sobreira L**. and **Marins M. (2009):** Molecular evaluation of the incidence of *Ehrlichia canis*, *Anaplasma platys* and *Babesia* spp. in dogs from Ribeirao Preto, Brazil. The Veterinary Journal.**179**: 145-148.

Schaefer J.J., Kahn J., Needham G.R., Rikihisa Y., Ewing S.A. and **Stich R.W. (2008):** Antibiotic clearance of *Ehrlichia canis* from dogs infected by intravenous inoculation of carrier blood. Annals of New York Academy of Science.**1149**: 263-269.

Schalm O.W. (2000): Schalm's Veterinary Hematology. In: Fieldman BG, Zinkl JG, Jain NC (Eds.). 5th ed. Lippincott Williams & Wilkins, Baltimore, MD 1344.

Sehdev A.E.S. and **Dumler J.S. (2003):** Hepatic Pathology in Human Monocytic Ehrlichiosis - *Ehrlichia chaffeensis* Infection. American Journal of Clinical Pathology.**119**:859-865

Shipov A., Klement E., Reuveni-Tager L., Waner T.and **Harrus S. (2008):** Prognostic indicators for canine monocytic ehrlichiosis. Veterinary Parasitology.**153**: 131-138.

Sirigireddy K.R. and **Ganta R.R. (2005):** Multiplex detection of Ehrlichia and Anaplasma species pathogens in peripheral blood by Real-Time reverse transcriptase-polymerase chain reaction. The Journal of Molecular Diagnostics.**7**: 308-316.

Singh M.H., Singh N.D., Singh C. and **Rath S.S. (2014)**: Molecular prevalence and risk factors for the occurrence of canine monocytic ehrlichiosis. Veterinari Medicina. **59**: 129-136.

Smith R.D., Hooks J.E., Huxsoll D.L. and **Ristic M. (1974):** Canine ehrlichiosis (tropical canine pancytopenia): survival phosphorus- 32- labelled blood platelets in normal and infected dogs. American Journal of Veterinary Research.**35**: 269-273.

Stiles J. (2000): Canine Rickettsial Infections. Veterinary Clinics North America Small Animal Practice.**30**:1135-1149.

Stockham S.L., Schmidt D.A. and **Tyler J.W. (1985):** Canine granulocytic ehrlichiosis dogs from central Missouri: a possible cause of polyarthritis. Veterinary Medical Review.**6**:3-5.

Stubbs C.J., Holland C.J. and **Relf J.S. (2000):** Feline ehrlichiosis. Compendium on counting Education for the Practicing Veterinarian.**22**: 307-318.

Stuen S. (2007): Anaplasma phagocytophilum- the most widespread tick-borne infection in animals in Europe. Veterinary Research Communications.**31**: 79-84.

Sumner J.W., Storch G.A., Buller R.S., Liddel A.M., Stockham S.L., Rikihisa Y., Messenger S. and **Paddock C.D. (2000):** PCR amplification and phylogenetic analysis of groESL Operon sequences from *Ehrlichia ewingii* and *Ehrlichia muris*. Journal of Clinical Microbiology.**38**:2746-2749.

Suto Y., Suto A., Inokuma H., Obayashi H. and **Hayashi T. (2001):** First confirmed canine case of *ehrlichia canis* infection in Japan. Veterinary Record.**148**:809-811.

Telford S.R., Dawson J.E., Katavolos P., Warner C.K., Kolbert C.P. and **Persing D.H. (1996):** Perpetuation of the agent of human granulocytic ehrlichiosis in a deer tick-rodent cycle. Proceeding of National Academy of Science. USA. **93**: 6209-6214.

Tsachev I., Gundasheva D., Kontos V., Papadogiannakis E. and **Denev S. (2013):** Haematological profiles in canine monocytic ehrlichiosis: a retrospective study of 31 spontaneous cases in Greece. Revue de Medecine Veterinaire.**164**: 327-330.

Unver A., Ohashi N., Tajjima T., Stich R.W., Grover D. and **Rikihisa Y. (2001a):** Transcriptional analysis of p30 Major Outer Membrane Multigene Family of *Ehrlichia canis* in dogs, ticks, and cell culture at different temperatures. Infection and Immunity.**69**: 6172-6178.

Unver A., Perez M., Orellana N., Huang H. and **Rikihisa Y. (2001b):** Molecular and antigenic comparison of *Ehrlichia canis* isolates from dogs, ticks, and a human in Venezuela. Journal of Clinical Microbiology.**39**:2788-2793.

Van Heerden J. (1982): A retrospective study on 120 natural cases of canine ehrlichiosis. Journal of South African Veterinary Association.**53**: 17-22.

Waddle J.R. and **Littman M.P. (1988):** A retrospective study of 27 cases of naturally occurring canine ehrlichiosis. Journal of American Animal Hospital Association.**24**: 612-620.

Waner T. and **Harrus S. (2000):** Canine monocytic ehrlichiosis. In: Recent Advances in Infectious Diseases. L.E. Carmichael (Ed.). International Veterinary Information Service.www.ivis.org. doc. No.A0108.0400.

Waner T., Harrus S., Bark H., Bogin E., Avidar Y. and **Keysary A. (1997):** Characterization of the subclinical phase of canine ehrlichiosis in experimentally infected beagle dogs. Veterinary Parasitology.**69**: 307-317.

Waner T., Harrus S., Weiss D.J., Bark H. and **Keysary A. (1995):** Demonstration of serum anti-platelet antibodies in experimental acute canine ehrlichiosis. Veterinary Immunology and Immunopathology.**48**:177-182.

Waner T., Leykin I., Shinitsky M., Sharabani E., Buch H., Keysary A., Bark H. and **Harrus S. (2000a):** Detection of platelet bound antibodies in beagle dogs after artificial infection with *Ehrlichia canis*. Veterinary Immunopathology.**77**:145-150.

Waner T., Strenger C. and **Keysary A. (2000b):** Comparison of clinic based ELISA test kit with the immunofluorescence test for the essay of *Ehrlichia canis* antibodies in dogs. Journal of Veterinary Diagnosis and Investigation.**12**:240-244.

Waner T., Rosner, M., Harrus, S., Naveh A., Zass R. and **Keysary A. (1996):** Detection of ehrlichial antigen in plasma of dogs with experimental acute *Ehrlichia canis* infection. Veterinary Parasitology.**63**:331-335.

Waner T., Harrus S., Jongejan F., Bark H., Keysary A. and **Cornelissen AWCA. (2001):** Significance of serological testing for ehrlichial disease in dogs with special emphasis of canine monocytic ehrlichiosis caused by *Ehrlichia canis*. Veterinary Parasitology**. 95**:1-15.

Wells M.Y. and **Rikihisa Y. (1988):** Lack of lysosomal fusion with phagosomes containing *Ehrlichia risticii* in P388 D1 cells: abrogation of inhibition with oxytetracycline. Infection and Immunity.**56**: 3209-3215.

Wen B., Rikihisa Y., Mott J.M., Greene R., Kim H.Y., Zhi N., Couto G.C., Unver A. and **Bartsch R. (1997):** Comparison of nested PCR with immunofluorescent-antibody assay for the detection of *Ehrlichia canis* infection in dogs treated with doxycycline. Journal of Clinical Microbiology.**35**: 1852-1855.

Woldehiwet Z. (2006): Anaplasma phagocytophilum in ruminants in Europe. Annals of the New York Academy of Sciences.**1078**: 446-460.

Woody B.J and **Hoskins J.D. (1991):** Ehrlichial diseases of dogs. Veterinary Clinics of North America Small Animal Practice.**21**: 75-98.

Yu X., McBride J.W. and **Walker D.H. (2007):** Restriction and expansion of Ehrlichia strain diversity. Veterinary Parasitology.**143**: 337–346.

Zhang L., Liu Y., Ni D., Li Q., Yu Y., Yu X., Wan K. Li D., Liang G., Jiang X., Jing H., Run J., Luan M., Fu X., Zhang J., Yang W., Wang Y., Dumler J.S. Feng, Z., Ren J. and **Xu J. (2008):** Nosocomial Transmission of Human Granulocytic Anaplasmosis in China. Journal of American Medical Association.**300**: 2263-2270.

3.6. Appendix I. The retrospective data capture sheet.

RETROSPECTIVE STUDY RECORD SHEET

Case#.............................. Date……………………………..

Patient……………………….. Owner…………………………………………

Breed…………….. Sex……………. Age……………………………………….

History…………………………………………………………………………………

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Findings…………………………………………………………………………………

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Blood smear…………………………… Diagnosis…………......................

Concurrent Diagnosis…………………......... Weight..............................

Treatment…………………………………………………………………………

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2nd Treatment
Date……………………….. Weight……………………………………

Response…………………………………………………………………………………

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