Development and validation of an *Ehrlichia canis* real-time PCR assay

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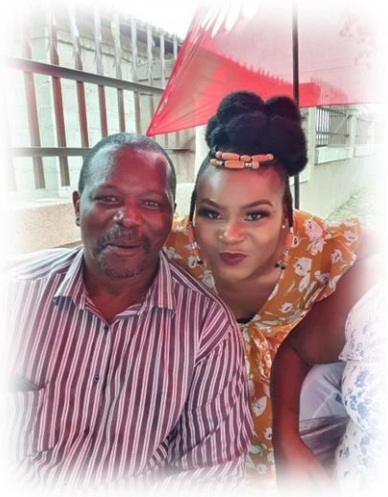
Dedication

To the one true God who is working in me, giving me the desire and the power to do what pleases him.

To my father the late Mathews Bhutshani Nkosi, thank you for your selflessness, love, support and words of encouragement. Your sacrifices and noble character will always echo in my heart. I am eternally grateful for having had you as a father. Heaven gained an angel, may your soul rest in eternal peace and let perpetual light shine upon you.



NKOSI DLAMINI wena wekunene, wena lowacedza
LUBOMBO NGEKUHLEHLETELA. Sidlubuladledle
sakalobamba, sona sitsi SIBADLA SIBE
SIBADLUBULISA. Samketi SINEMBOVU KuMALANGENI
Wena longayidli imvu lemnyama,
Wesaba emafinyila nemcondvo.
wena losidvwaba silutfuli, singabantolo sifute
ekhaboNYOKO ,NKOSI wenalowabophela LOKUHLE EMTFUNTINI wentela bafati
BETEKUHAWUKELA.
NKOSI!!! DLAMINI!!!! Awucedvwa Ngwane!!!!

Declaration

I, Nokuzola Faith Nkosi, declare that the work presented in this dissertation, which I hereby submit for the degree Masters in Veterinary Science Tropical Diseases at the University of Pretoria, Department of Veterinary Tropical Diseases, was executed by me under the supervision of Professor Melvyn Quan and the co-supervision of Professor Marinda Oosthuizen. This is the first presentation of this work for degree compliance at this or any other tertiary institution, sources of information have been acknowledged accordingly.

Date

Signature

Abstract

Development and validation of an *Ehrlichia canis* real-time PCR assay

by

Nokuzola F. Nkosi

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Department: **Department of Veterinary Tropical diseases**

Degree: **MSc (Veterinary Science Tropical Diseases)**

Ehrlichiosis is caused by a pleomorphic gram-negative bacteria and is an important zoonotic tick-borne disease, with a potential to be fatal. This bacterium occurs worldwide and species affected by it include humans, domestic and wild animals. Canine monocytic ehrlichiosis develops over a period of about 8-20 days and may progress from an acute phase to subclinical and chronic disease stages. Although direct detection of the bacterial antigen by ELISA has been used successfully to diagnose the disease, a challenge remains in dogs where co-infection of infectious agents is common due to pathogens being transmitted by the same vectors. Cross-reactivity of the serology assays makes it difficult to make species-specific diagnoses. A more sensitive and reliable molecular technique that can detect and identify pathogens at species level is needed to enhance disease diagnosis.

In this study, we developed a real-time PCR assay that employs group-specific primers and an *Ehrlichia canis* TaqMan® minor groove binder probe. The group-specific primers targeted the conserved region of the 16S rRNA gene and the forward primer included redundant base pairs to accommodate other species in this genus. The primer and probe concentrations were optimised to 200 nM and 250 nM respectively. The efficiency of the assay was 93%. The assay was *E. canis* specific when tested against other canine and livestock pathogens, as no cross-reactivity was observed. The 95% limit of detection was 33 *E. canis* plasmid copies/µl of blood (95% confidence interval: 23 - 58). Consistent repeatability was observed, where the inter-run standard deviation (SD) ranged between 0.33 - 1.29 and the intra-run SD 0.04 - 1.14. The results for Reverse Line Blot (RLB) hybridization assay and the TaqMan® MGB real-time PCR assay results were compared and found to be in agreement with an exception of three samples out of 121. Diagnostic validation was performed on field samples, the sensitivity of the TaqMan® MGB real-time PCR assay was 90% and the specificity was 92%. This assay will be a useful tool for the early diagnosis of *E. canis* and this will aid in timely treatment.

Key words: Dogs, Tick-borne disease, *Ehrlichia canis*, TaqMan®

Research outputs

Conference oral presentation: N.F. Nkosi, M.C. Oosthuizen, M. Quan. 2018. Development of a real time PCR assay to differentiate *Ehrlichia/Anaplasma* spp. in dogs. The 47th Parasitological Society of Southern Africa (PARSA) Congress at Tshipise Forever Resort, Limpopo, South Africa from the 16-18 September 2018.

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Ethics statement

The Department of Veterinary Tropical Diseases (DVTD), University of Pretoria, Faculty of Veterinary Science laboratory facilities and equipment were used to execute this research.

Reagents were purchased from different suppliers according to the need of the project design.

This study obtained approval from the University of Pretoria's Animal Ethics Committee (project no. V099-17) (Appendix 8.6), and Section 20 clearance (ref. 12/11/1/1/6) (Appendix 8.7) from the Department of Agriculture, Forestry and Fisheries for the research described in this work.

I, Nkosi N.F (student no. u04906480) declare that ethical standards required in terms of the University of Pretoria's Code of Ethics for Researchers and the Policy guidelines for responsible research were followed accordingly.

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Abbreviations used

%	Percentage
°C	Degrees Celsius
CME	Canine monocytic ehrlichiosis
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DVTD	Department of Veterinary Tropical Diseases
EDTA	Ethylenediaminetetraacetic-acid
ELISA	Enzyme-linked immunosorbent assay
IFA	Indirect Immunofluorescence Assay
MAHC	Mamelodi Animal Health Clinic
$MgCl_2$	Magnesium chloride
ml	Millilitre
OVAH	Onderstepoort Veterinary Academic Hospital
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PK	Proteinase K
RLB	Reverse Line Blot
rRNA	Ribosomal Ribonucleic Acid
spp	Species
µl	Microlitre
µM	Micromolar
UP	University of Pretoria

Chapter 1
Introduction

Ehrlichiosis is an important zoonotic tick-borne disease affecting dogs world-wide (Mylonakis and Theodorou, 2017, Stich et al., 2008, Batmaz et al., 2001). It is caused by a gram-negative bacterium in the order *Rickettsiales*, family *Anaplasmataceae* and genus *Ehrlichia/Anaplasma* (Mylonakis et al., 2019, Dumler et al., 2001, Mylonakis and Theodorou, 2017, Rikihisa, 1991). The genus *Ehrlichia* consists of several species but only the following species are known to affect dogs: *Ehrlichia ewingii, Ehrlichia chaffeensis* and *Ehrlichia canis* (Little, 2010, Walker and Dumler, 1996). Dogs infected with *E. canis* develop canine monocytic ehrlichiosis (CME) a significant infectious disease of canines. *Ehrlichia ewingii* causes canine granulocytic ehrlichiosis and *E. chaffeensis* causes human monocytic ehrlichiosis (Cohn, 2003). Transmission of these pathogens occurs by means of a tick vector in the family *Ixodidae* and occasionally by medical procedures that involve blood transfusions, organ transplant or bone marrow transplants (McQuiston et al., 2000). The spread of each pathogen is dependent on the availability of the vectors and mammalian reservoir hosts (Hinrichsen et al., 2001).

Canine monocytic ehrlichiosis (CME) presents with non-specific clinical signs (Harrus et al., 1997b, Buhles et al., 1974). The intensity of the symptoms in an infected animal is influenced by the inoculum size, the bacterium species and strain and host immunity (Harrus et al., 1997b, Gaunt et al., 1996). Multiple infection with more than one rickettsial pathogen is common, due to shared vector or concurrent exposure to multiple tick vectors (Kordick et al., 1999). As a result, diagnosis can be complicated. Current available diagnostic tests are non-specific nor consistent, some tests are expensive and labour intensive (Parola et al., 2001).

There are various test available on the market to diagnose ehrlichiosis. They include serological diagnosis such as western immunoblotting, enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence assay (IFA), polymerase chain reaction (PCR) and cell culture. Some of these tests have various shortcomings, like lack of sensitivity and specificity, some are laborious to perform and require skilled personnel to analyse the results (Sykes, 2014). PCR and sequencing are sensitive and specific methods for pathogen characterization (Iqbal et al., 1994).

In this study we developed and validated a species-specific real-time PCR assay to detect *E. canis* in dogs. This assay can be performed quickly, is reliable, sensitive and specific, therefore aiding with timely diagnosis and treatment. This will also help with co-infection impact evaluation once multiplexed with in-house *Babesia rossi* and *Babesia vogeli* real-time PCR assay, as well as inhibiting spread of disease through trade and therapeutics studies.

1.1 Study aim

To develop and validate a real-time PCR assay to detect *Ehrlichia canis* in dog blood.

1.2 Objectives

- Identify conserved regions and species-specific regions, design primers and probes for TaqMan® MGB real-time PCR assay.
- Optimise the TaqMan® MGB real-time PCR assay.
- Validate the TaqMan® MGB real-time PCR assay.
- Develop a standard operating procedure to execute the *E. canis* TaqMan® MGB real-time PCR assay.

Chapter 2
Literature review

2.1 *Ehrlichia* species

Ehrlichia species are infectious, highly pleomorphic, obligate intracellular gram-negative bacteria that infect humans, wild animals and several domestic animal species, namely cats, horses and dogs, and cause ehrlichiosis. These bacteria are transmitted by vectors and unlike most gram-negative cocci, they do not cause endotoxemia. Ehrlichiosis causes severe clinical illness in South African dogs, making it one of the most important tick-borne disease (Rautenbach et al., 1991).

Ehrlichia species belong to the order *Rickettsiales*, family *Anaplasmatacae* (Dumler *et al*., 2001). They were characterised initially according to the type of cells infected viz. granulocytes, monocytes, lymphocytes or thrombocytes (Rikihisa, 1991), but improved molecular techniques allowed for the use of sequence homology of ribosomal RNA (rRNA) genes to establish the genetic relatedness of a number of organisms. This has led to the reclassification of *Ehrlichia* species: *Ehrlichia bovis*, *E. platys*, *E. phagocytophila* also known as *E. equi* or human granulocytic ehrlichial agent (HGE) were grouped in the *Anaplasma* genus. In the *Ehrlichia* genus, *Cowdria ruminantium* was included as *E. ruminantium* with the existing species, namely, *E. canis*, *E. chaffeensis*, *E. ewingii.* The genus *Neorickettsia* was expanded to include *Ehrlichia risticci* and *Ehrlichia sennestu*. *Wolbachia pipientis* remained the sole member of *Wolbachia* genus (Dumler *et al*., 2001). The various *Ehrlichia / Anaplasma* species affecting dogs are listed in Table 2.1.

Ehrlichia chaffeensis is a potentially fatal mononuclear cell human pathogen closely related to *E. canis* and transmitted by *Amblyomma americanum* and *Rhipicephalus sanguineus* vectors (Ndip et al., 2010, Cohn, 2003). It was first described as a disease in the late 1980s in the United State of America (USA) but officially became a reportable disease in 1999 (Maeda et al., 1987). People infected with *E. chaffensis* may manifest with fever, headache, myalgia and more severe signs include vomiting, epistaxis, lymphadenopathy and anterior uveitis (Walker and Dumler, 1996). A study conducted by Breitschwerdt et al., (1998) confirmed that dogs can develop severe disease manifestation from *E. chaffeensis* natural infection (Dawson

et al., 1996). *Ehrlichia chaffeensis*-like species, with about 97% identity using the *Ehrlichia* genus thio-oxidoreductase protein gene (*dsb*A), have been reported in South Africa (Iweriebor *et al*., 2017) and there is serological evidence of *E. canis* and *E. chaffeensis* in dogs from Bloemfontein, South Africa (Pretorius and Kelly, 1998), however serological cross reactivity may occur between these organisms.

Ehrlichia ewingii is infectious to both humans and dogs and was first described in dogs with a febrile illness in 1971 (Ewing et al., 1971). It is transmitted by *A. americanum* and causes granulocytic ehrlichiosis, which has a very low fatality rate (Cohn, 2003). Symptoms of infection are similar to those of *E. canis* but milder. *Ehrlichia ewingii* has been detected in domestic dogs in Cameroon by PCR (Williams *et al*., 2014). There is no evidence of the presence of this pathogen in South Africa.

Anaplasma phagocytophilum causes human granulocytic anaplasmosis (HGA) and canine anaplasmosis. It is characterized by an acute febrile illness and is transmitted by *Ixodes scapulari, I. ricinus* and *I. pacificus* (Iweriebor et al., 2017, Ogden et al., 2003, Richter et al., 1996). An organism closely related to *A. phagocytophilum* has been reported in dogs from South Africa (Mtshali et al., 2017, Inokuma et al., 2005, Matjila et al., 2008), and referred to as *Anaplasma* sp. South Africa dog and subsequently also from Zambia: *Anaplasma* sp. Zam dog.

Anaplasma platys (formerly known as *E. platys*) is distributed worldwide and causes canine infectious cyclic thrombocytopenia, which is platelet-specific (Carvalho et al., 2017). It is transmitted by the vector *R. sanguineus*, which also transmits *E. canis.* This pathogen has been detected in ticks sampled from ruminants in Free State, South Africa (Berggoetz et al., 2014). Canines are the natural hosts of *A. platys.*

Ehrlichia canis was first identified in 1935 in Algeria and causes canine monocytic ehrlichiosis (CME) in dogs and members of the *Canidae* family. Canine monocytic ehrlichiosis/ anaplasmosis can also be caused by other *Ehrlichia* species such as *E. chaffeensis, E. ewingii, A. phagocytophilum*, and *A. platys*. These organisms are distributed worldwide in Europe, Asia, Africa and America, but are more prevalent in the sub-tropical and tropical regions of the world, where the biological vector *R. sanguineus* thrives (Fourie et al., 2013).

Canine monocytic ehrlichiosis infects all dog breeds, but German shepherds are more susceptible, presenting with more severe clinical signs and higher morbidity and mortality compared to other breeds (Nyindo et al., 1980). In the 1970's, the disease resulted in hundreds of American military German shepherd dog deaths. Clinical findings remain inconsistent due to different strains of the organism that vary in virulence, the immunity of the host, age, breed and coinfection status (Gal et al., 2007, Cohn, 2003).

The first *E. canis* human case description was reported in Venezuela (Unver et al., 2001, Perez et al., 1996).

In South Africa, the pathogens that cause ehrlichiosis infections are not well characterized. *Ehrlichia canis* has been detected by serological and molecular methods (Mtshali et al., 2017, Matjila et al., 2008, Pretorius and Kelly, 1998).

Table 2.1 Characteristics of *Ehrlichia* and *Anaplasma* species affecting dogs.

Name	Natural host/s	Biological vector/s	Cell tropism	Disease caused
E. chaffeensis	Human, dogs	*A. americanum/R. sanguineus*	Mononuclear	Human monocytic ehrlichiosis
E. ewingii	Human, dogs	*A. americanum*	Granulocytes	Canine granulocytic ehrlichiosis
A. platys	Dogs	*R. sanguineus*	Platelets	Cyclic thrombocytopenia
A.phagocytophilum	Dogs, people, horses, small ruminants	*Ixodes* species including *I. Pacificus, I. ricinus* and *I. scapularis*	Granulocytes	Human granulocytic anaplasmosis, Canine granulocytic anaplasmosis
E. canis	Dogs	*R. sanguineus*	Mononuclear	Tropical pancytopenia, Canine monocytic ehrlichiosis

2.2 Transmission of infection

An extensive range of pathogens are transmitted by ticks, including bacteria, rickettsiae viruses and protozoa. These pathogens cause consequential and potentially fatal disease in humans and animals worldwide (Kim et al., 2006). *Rhipicephalus sanguineus*, the brown dog tick, is the dominant vector of *E. canis* (Gal et al., 2008, Wen et al., 1997). However, there are other tick species in which *E. canis* DNA has been detected from and they include *Ixodes ricinus, Haemaphysalis* spp. ticks, *Dermacentor* spp. ticks, *D. variabilis* and other *Rhipicephalus* spp. (Johnson et al., 1998).

The distribution of *R. sanguineus* is worldwide in the warmer regions of Asia, Africa, Europe, and the America. An increase in tick activity is usually observed during the warmer months.

In South Africa this occurs from October/November to March/April, but even during autumn and winter, a small number of adult ticks will still be present (Jacobs et al., 2001).

The Ixodid tick life cycle consist of four phases: egg, larva, nymph and adult. Each phase requires a blood meal except for the egg which hatches to a larvae, as shown in Figure 2.1 (Sykes, 2013, Horak et al., 2002). The infection is obtained by the tick when a larvae, nymphs or adult feed on an infected dog (Bremer et al., 2005, Johnson et al., 1998). Pathogens are transferred transstadially and intrastadially but not transovarially (Neer and Harrus, 2006, Bremer et al., 2005, Groves et al., 1975). The tick, therefore, does not act as a reservoir of the pathogen. The infection may last for several months in an infected tick and can be transmitted by adult ticks that have over-wintered as infected nymphs, before converting to adults in spring (Neer and Harrus, 2006). *Ehrlichia canis* infection in ticks is restricted to the midgut and salivary glands (Fourie et al., 2013, Smith et al., 1976).

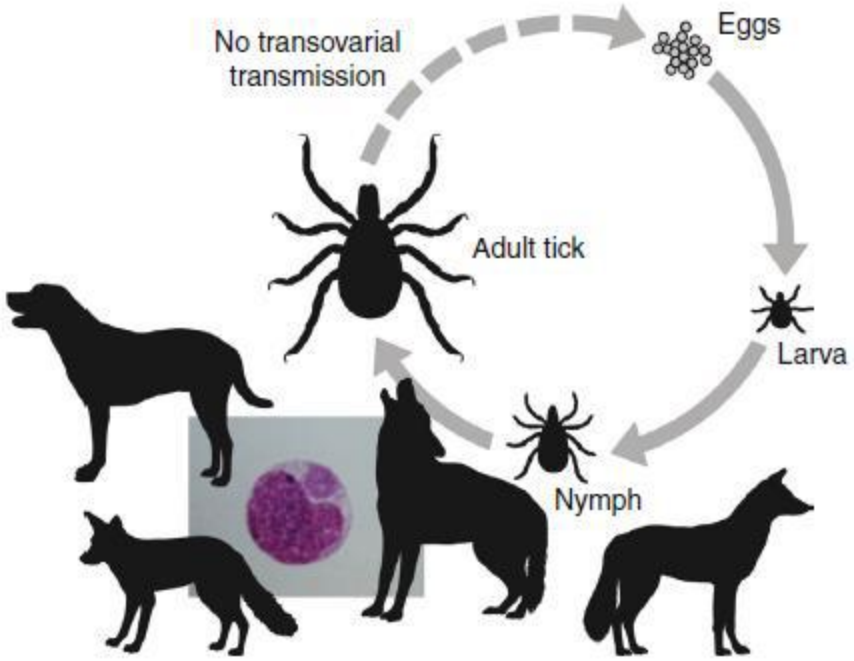

Figure 2.1 Transstadial transmission of *Ehrlichia canis* in *Rhipicephalus sanguineus* ticks (Sykes, 2013).

In South Africa, *R. sanguineus* was reported, in addition to other species, to feed on humans (Horak et al., 2002). Co-existence of a number of pathogens in dogs may be caused by the ability of one tick vector to transmit a number of different pathogens e.g. *R. sanguineus* can transmit *E. canis*, *E. ewingii, Babesia canis, B. gibsoni* and *A. platys* (Little, 2010, Cohn, 2003, McBride et al., 2001).

2.3 Pathogenesis of canine monocytic ehrlichiosis (CME)

The target cell of this pathogen are monocytes and macrophages of dogs (Stich et al., 2002). The bacterium multiplies by binary fission within mononuclear phagocytes vacuoles and infection of cells occurs when an infected cell burst. *Ehrlichia canis* spreads in the host cells through different stages such as adhesion, internalisation, intracellular proliferation, exocytosis and intercellular spreading (Harrus, 2015). Pathology is a result of the direct effects of the pathogen and consequential indirect immune mediated mechanisms.

The disease has an incubation period of about 8-20 days, which develops into three successive stages of CME: acute phase (2–4 weeks duration), subclinical (months to years) and chronic disease stages, even though the difference between these stages is not clear-cut in dogs with naturally-occurring disease (Harrus, 2015). Manifestation of *E. canis* infection relates to a broad spectrum of clinical signs, considering the type of strain causing infection, counter response of the host immunity, disease stage and coinfections.

2.4 Prevalence of *E. canis* in South Africa

A study conducted in Bloemfontein, South Africa reported 42% of seroprevalence in dogs when tested with IFA, this was comparable to 33-68% which was obtained in a study conducted in a neighbouring country, Zimbabwe (Pretorius and Kelly, 1998, Matthewman et al., 1993a, Matthewman et al., 1993b). Molecular evaluation on canine blood samples was conducted and it was found that 2% of domestic dogs in South Africa was co-infected with *Babesia rossi* and *E. canis* (Matjila et al., 2008). Similar results were obtained in another study which was conducted at Onderstepoort on dogs that were brought in for veterinary care to the Onderstepoort Veterinary Academic Hospital (OVAH), Gauteng, South Africa, 1.5% of the dogs were co-infected with *B. rossi* and *E. canis*, and 1.3% had *B. vogeli* and *E. canis* co-infection (Rautenbach et al., 2018).

2.5 Clinical signs

Infected animals present with non-specific clinical signs. Shared vector or concurrent exposure to multiple tick vectors is another component that lead to non-specific clinical signs

due to infection with more than one rickettsial or other arthropod-borne pathogens (Sykes, 2014).

The acute phase persists for about 1- 4 weeks, which dogs can recover spontaneously from, by eliminating the infection or remain infected sub-clinically, an asymptomatic phase which can last for months to years with mild thrombocytopenia as the only suggestion of infection.

A suspicious case in the acute phase would include symptoms such as fever, headache, lethargy, depression, splenomegaly, anorexia, swollen lymph glands, weight loss, thrombocytopenia, anaemia, hypergammaglobulinaemia, pancytopenia, haemorrhage, epistaxis, malaise, myalgia, arthralgia, nausea and/or vomiting (Fourie et al., 2015, Harrus, 2015).

The components of the chronic phase induction remain unspecified and the percentage of dogs entering this phase is unknown due to naturally and experimentally-infected remaining healthy for several years post exposure. Manifestation of chronic phase of *E. canis* is characterized by lethargy, lack of appetite, bleeding abnormalities, mucosal pallor, pyrexia, epistaxis, chronic weight loss, lymphadenopathy, splenomegaly, dyspnoea, anterior uveitis, retinal haemorrhage and detachment, polyuria/polydipsia and peripheral oedema (Mylonakis et al., 2004, Harrus et al., 1997a). Chronic infections permits canids to serve as reservoir for infections.

2.6 Treatment and prevention

Treatment of CME remain a therapeutic challenge to primary care givers, due to lack of controlled therapeutic trials and arguable documentation of therapeutic elimination of CME. Documented post-treatment information is lacking. No vaccines are available for ehrlichiosis. There are numerous drugs that have been investigated to treat *E. canis* infection, such as tetracyclines, macrolides, fluoroquinolones, chloramphenicol, rifampicin and imidocarb dipropionate, which are unsatisfactory except for tetracyclines and chloramphenicol. Due to the potential toxicity of chloramphenicol, the use of this drug has been stopped and should only be used in cases where tetracyclines cannot be used (Harrus, 2015, Breitschwerdt et al., 1998). The rapid development of antibiotic resistance is a concern, with the likelihood that current antibiotic treatment will be not effective to treat ehrlichial and rickettsial infections in the future (Ventola, 2015).

Doxycycline inhibits protein synthesis, like other tetracyclines, to prevent bacterial proliferation. The antibiotic is administered orally, 10 mg/kg every 24 hours for 28 days in infected animals (Little, 2010, Harrus et al., 2004, Cohn, 2003). To enable careful administration of antibiotics for antimicrobial resistance prevention, it is advisable to monitor dogs clinically, haematologically and serologically at least biannually, instead of antibiotic usage on a dog that might have naturally immunologically cleared the infection (Neer et al., 2002). The majority of dogs and cats recover fully after treatment with doxycycline or tetracycline at the acute stage and subclinical stage, however, the chronic stage is incurable and its prognosis is severe (Harrus, 2015, Harrus and Waner, 2011, Little, 2010, Harrus et al., 2004, Cohn, 2003, Murphy et al., 1998). Other prevention methods include tick control by application of effective products on the dog and its environment to decrease tick population.

2.7 Diagnosis of infection

Canine monocytic ehrlichiosis diagnosis can be difficult, due to the non-specific symptoms of the disease. Infection should be suspected when the animal has been to an endemic area or been exposed to the vector, together with presenting clinical signs (Cohn, 2003). There are a number of established techniques for ehrlichiosis diagnosis, which include haematology, cell culture, serology (such as ELISA and IFA) and molecular techniques, e.g. PCR (Little, 2010, Cohn, 2003).

2.7.1 Haematology

A blood smear can be made to visualize the morulae within infected cells under a light microscope (Figure 2.2) (Harrus and Waner, 2011, Hildebrandt et al., 1973). A complete blood count can be performed to indicate haematological irregularities, such as leucopaenia and thrombocytopaenia (Harrus and Waner, 2011, McBride et al., 2001), but low parasitaemia can make it difficult to detect an infection. This method also requires skilled personnel to accurately identify cell infection, but lacks sensitivity and specificity. An extensive range of clinical presentations creates difficulties in diagnosis and clinical management.

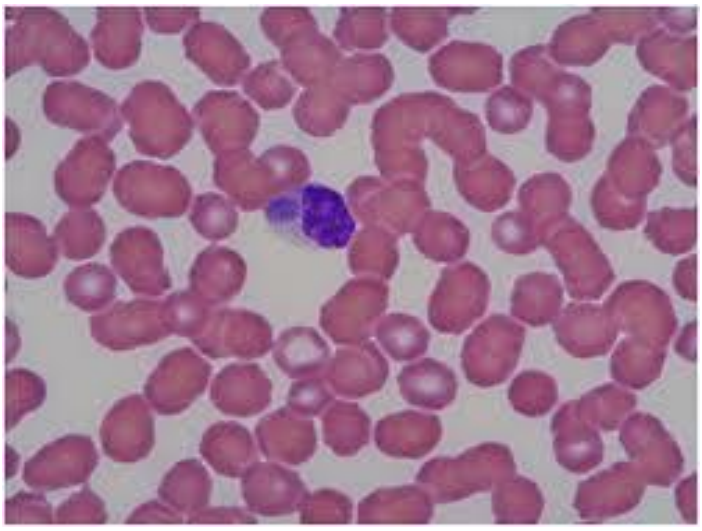

Figure 2.2 A microscopic image of *E. canis* morulae in the cytoplasm of a monocyte (x100) (Sainz et al., 2015).

2.7.2 Cell culture

Isolation of *E. canis* using cell culture techniques was found to be the most sensitive and reliable diagnostic method, however, this method is time consuming, laborious and requires specialized cell culture techniques and facilities, which adds to the cost therefore not practical for routine diagnosis (Iqbal et al., 1994). The isolation method also requires specific laboratory equipment and trained personnel.

2.7.3 Serology

There are several serological methods that are considered valuable for diagnosis of CME. Indirect immunofluorescence assay testing, ELISA technology, and western blotting have been used to diagnosis ehrlichiosis (Harrus et al., 2002, Waner et al., 2000). Indirect immunofluorescence assay, the serological gold standard, which tests for IgG antibodies in a serum sample has been the most widely used test to indicate exposure to *E. canis* (Harrus and Waner, 2011, Ristic et al., 1972). It is difficult to detect infection at an early stage using the IFA test because antibodies are usually absent during the first two weeks of infection and in immunocompromised patients. Another shortcoming of the IFA test is its non-specific detection, due to antigenic cross-reactivity with closely related species, resulting in false-positive results (Nazari et al., 2013, Harrus et al., 2002, McBride et al., 2001, Waner et al., 2001, Ristic et al., 1981). It cannot discriminate between *E. canis, E. ewingii, E. chaffeensis* and *E. ruminantium* antibodies (Cardenas et al., 2007). Cross-reaction between *E. canis* antibodies

and *A. phagocytophilum* antigen has also been shown to develop over a period of 55-150 days post infection (Waner et al., 1998). The assay is highly sensitive to detect the occurrence of exposure, but cannot establish the existing infection status or disease clearance after treatment, since animals remain IFA positive for a longer period after clearance of the pathogen (Iqbal and Rikihisa, 1994).

The 3Dx SNAP (IDEXX Laboratories, Inc.) is an in vitro diagnostic test which can detect antibodies of *Ehrlichia canis* using artificial peptides resulting from the main immunodominant *E. canis* proteins *P30* and *P30*-1 in canine whole blood, serum or plasma (Harrus et al., 2002). This test was deemed inadequate for interpretation of clinical relevance when used alone. Other test such as platelet counts and molecular results should be used in combination with it (Hegarty et al., 2009).

Western immunoblot has been used to differentiate and characterize various organisms causing ehrlichiosis. It is a more specific serological method due to its ability to characterize the pathogen by finger printing its immunogenic protein profile. Normally a similar pattern is retrieved amongst various *E. canis* strains, however, unique main proteins were discovered in western blot patterns in sera from Italy and Zimbabwe, reinforcing the probability of antigenic diversity among *E. canis* from diverse parts of the world (Hegarty et al., 1997).

Serological tests have disadvantages such as cross reactivity with closely related species.

2.7.4 Molecular techniques

PCR and sequencing are sensitive and specific methods for pathogen detection and characterization. The utilization of PCR in molecular diagnostics has grown to the point where it is now acknowledged as the standard method for detecting nucleic acids from a number of sample and microbial types (Mackay, 2004).

PCR assay efficiency is described as the doubling of the target DNA that is replicated in one cycle (Alvarez et al., 2007). Presuming that the concentration of the PCR doubles every cycle throughout the exponential phase the ideal amplification efficiency equals 100%, however, exhaustion of PCR reaction reagents, enzyme activity decline and competition with other PCR products cause a gradual decline of amplification efficiency to zero (Zhang et al., 2015, Wong and Medrano, 2005).

Real-time PCR permits quantitative data collection throughout the PCR process as it occurs, amplification and detection are achieved in a single step. The number of cycles needed for the fluorescent signal to cross the threshold is defined as C_T (Heid et al., 1996). A variety of different fluorescent chemistries make it achievable to correspond PCR product concentrations to fluorescent potency (Wong and Medrano, 2005, Higuchi et al., 1993). It has four stages namely the linear ground stage, early exponential stage, log-linear/exponential stage, and plateau stage (Tichopad et al., 2003). Real-time PCR assay uses the TaqMan® minor groove binder (MGB) probe integrate a non-fluorescent quencher (NFQ) to quench the signal of the fluorescent dye label on the 5' of the probe. The quencher is known to turn down the reporter fluorescence intensity by fluorescence resonance energy transfer (FRET) when the probe is intact. This produces a lower background signal than with non-MGB NFQ, which in turn increase sensitivity to single base mismatch and precision. The MGB increases the probe melting temperature (T_m), resulting in highly stable probe-target duplexes with the single-stranded DNA targets. This allows hybridization assays to have highly specific probes that are shorter than standard probes and provide exceptional sequence discrimination and flexibility to accommodate more targets (Kutyavin et al., 2000).

A number of assays targeting different *Ehrlichia* genes, like 16S rRNA, *p28*, *p30*, *groESL*, disulfide bond formation protein (*dbs*) and *VirB9* have been described (Bell and Patel, 2005, Stich et al., 2002, Gusa et al., 2001). Published multiplex assays include a triplex qPCR assay to detect *E. chaffeensis, E. ewingii*, and *E. canis*, using genus-specific primers targeting the *dsb* gene and species-specific TaqMan® probes (Doyle et al., 2005), and a multiplex real-time qPCR assay for *E. canis* (targeting 16S rRNA) and *Babesia vogeli* (targeting the heat shock protein 70) (Peleg et al., 2010) and a multiplex qPCR assay for detecting *E. chaffeensis, E. canis, A. phagocytophilum* and *A. platys* amplifying 16S rRNA (Sirigireddy and Ganta, 2005).

The nested PCR assay has been used for detection of *Ehrlichia* spp., however chances of contamination which may lead to false positive results are high when using this method and it is also more laborious and costly (Yabsley et al., 2008).

The reverse line blot hybridization assay (RLB) is another molecular biology assay used to detect multiple pathogens. Most assays are based on the detection of the 16S rRNA and p30 genes (Peleg et al., 2010, Bell and Patel, 2005, Stich et al., 2002). A RLB hybridization assay based on the 16S rRNA gene with species-specific probes to detect *Anaplasma* and *Ehrlichia*

species was established. Using an *E. canis* probe previously described by Schouls et al., (1999) this assay could detect *Ehrlichia ovina*, however, the sensitivity of the assay is unknown but all species could be detected by their relevant species-specific probes with no cross-reactivity (Bekker et al., 2002). Another RLB hybridization assay targeting the 18S rRNA for *Theileria* and *Babesia* which can co-exist with *Anaplasma and Ehrlichia* has been developed (Gubbels et al., 1999). The RLB hybridization assay is very laborious and time consuming to perform resulting in a slow turnaround time.

Advantages of molecular detection are an early diagnosis of the disease before antibodies are produced and higher sensitivity. PCR indicates presence of the pathogen, instead of antibodies to the pathogen, which shows previous exposure. New species detection and identification of closely related *Ehrlichia* spp. using group-specific primers and sequencing can be achieved by using real-time PCR (Nazari et al., 2013, Iqbal et al., 1994). Real-time PCR has caused broader recognition of PCR because it is more rapid, sensitive and reproducible, while the risk of carryover contamination is lessened (Mackay, 2004).

2.8 Real-time PCR validation following OIE guidelines

Validation is a route that defines the suitability of an assay which has been appropriately developed, optimised and standardised, for an intended purpose. Assay validation route comprises of numerous phases but an assay that has accomplished the first three phases of the validation pathway together with performance characterisation, can be defined as validated for the original anticipated purpose. The three phases of validation are phase 1 - analytical characteristics (analytical specificity and sensitivity), phase two - diagnostic characteristics (diagnostic specificity, sensitivity and cut-off) and phase 3 - reproducibility. laboratory and field assays validation should be done for the targeted species. It is compulsory to monitor the assay performance under regular conditions to retain validated status. The assay may be deemed unsuitable for its intended purpose should it no longer yield results consistent with the original validation data (OIE-Terrestrial-Manual, 2009).

Chapter 3
Materials and methods

3.1 Sample collection

This study involved the use of blood samples obtained from dogs suspected to be infected with ehrlichiosis at the University of Pretoria's (UP) Onderstepoort Veterinary Academic Hospital (OVAH) and Mamelodi Animal Health Clinic (MAHC). Dog owners signed consent forms (Appendix 8.2) to allow the use of samples for research purposes. Veterinarians working at the hospital restrained the dogs and collected blood (2 – 5 ml) in ethylenediaminetetraacetic acid (EDTA) anticoagulant via venepuncture.

Suspicious cases of ehrlichiosis were based on the following inclusion criteria disregarding sex, dogs > 5 kg and > 3 months of age that presented with either non-regenerative anaemia (pale mucous membranes, low haematocrit and lack of regeneration on a peripheral blood smear), thrombocytopaenia on a peripheral blood smear, easily palpable lymph nodes, large spleen, chronic history of illness (sometimes with weight loss), epistaxis, uveitis, pyrexia and leukopaenia. OVAH blood samples (n = 5) and MAHC blood samples (n = 9) were collected specifically for this study from dogs showing symptoms in our inclusion criteria for ehrlichiosis.

The study protocol was approved by the UP's Animal Ethics Committee (V099-17) (Appendix 8.6) and a South African Department of Agriculture, Forestry and Fisheries section 20 approval (ref. 12/11/1/1/6) (Appendix 8.7).

The *Ehrlichia canis* TaqMan® MGB real-time PCR assay that was developed was tested on a total of 121 samples (Appendix 8.1). The samples tested were diagnostic canine blood samples (n = 52) from the Department of Veterinary Tropical Diseases (DVTD) biobank submitted to DVTD from OVAH for testing for haemoparasites due to suspicion of haemoparasites, or as part of a diagnostic panel. Samples from Namibia (n = 55) were submitted to DVTD for haemoparasites testing due to suspicion of an infection, blood samples from Namibia were spotted on Whatman® FTA cards. Other blood samples tested were from OVAH blood samples (n = 5) and MAHC blood samples (n = 9).

3.2 TaqMan® MGB real-time PCR assay design

Ehrlichia canis 16S rRNA gene sequences were downloaded from GenBank® (www.ncbi.nlm.nih.gov/genbank) and aligned with MAFFT online version (https://mafft.cbrc.jp/alignment/server), set on default settings (Katoh et al., 2017). The *E. canis* strain Oklahoma GenBank® sequence with accession number NR_118741 was used as a reference sequence. Sequence alignment analysis and editing was performed with BioEdit Sequence Alignment Editor (Hall, 1999). Identical sequences were identified and collapsed with the aid of DAMBE (Xia, 2018).

A set of group-specific primers were designed along with species-specific TaqMan® minor groove binder (MGB) probes (Table 3.1) using Primer Express® version 3.0.1 (Applied Biosystems, USA). The forward included redundant nucleotides, due to differences amongst the different species sequences in the *Ehrlichia*/*Anaplasma* genus. A region specific to each species was selected as probe. Probes for *E. canis, E. chaffeensis, E. ewingii, Anaplasma platys, A. phagocytophilum* and *Anaplasma* sp. South Africa dog were designed but for this study only *E. canis* probe was tested and validated. To test for non-specific binding *in silico*, a nucleotide Basic Local Alignment Search Tool (BLASTn) screening was performed for both the primers and probe on NCBI web site (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The probes were labelled with a fluorescent reporter dye FAM (6-carboxyfluorescein) at the 5'-end and a nonfluorescent quencher-minor groove binder (NFQ-MGB) attached to the 3'-end.

Table 3.1 Sequence and characteristics of the group-specific *Ehrlichia / Anaplasma* forward / reverse primers (F/R) and *Ehrlichia / Anaplasma* species specific probes (P) targeting the 16S gene.

Name	Start	Stop	Primer sequence (5'-3')	Length	TM (°C)	% GC
AnapEhrlichia_F	23	44	AGCYTAACACATGCAAGTCGAA	22	59	45
AnapEhrlichia_R	82	103	TTACTCACCCGTCTGCCACTAA	22	58	50
E. canis_P	60	76	AGCCTCTGGCTATAGGA	17	69	53
A. platys_P	53	76	TGTCGTAGCTTGCTATGAT	19	69	42
E. chaffeensis_P	45	63	GGACAATTGCTTATAACC	18	68	39
E. ewingii_P	46	68	AACAATTCCTAAATAGTCTCTG	22	69	32
A. phagocytophilum_P	44	65	CGGRTTATTCTTTRTAGCTTG	21	68	38
A. sp. South Africa dog _P	50	71	ATCTTTGTAGCTTGCTACGAA	21	68	38

3.3 Primer and probe optimization

The dry stock of primers (Integrated DNA Technologies, USA) was resuspended in 100 µM stock concentration with Tris-EDTA (TE buffer). Dilutions to 20 µM working stocks aliquots

were made with ultrapure 18.2 MΩ.cm water (Elix® Essential 5 and Synergy® water purification systems, Merck). The probe (ThermoFisher Scientific, USA) was resuspended with Tris-EDTA (TE buffer) to 100 µM and further dilutions to 20 µM working stock aliquots were prepared with the same diluent. All stocks prepared were stored at -20°C until use.

The primer concentration was optimized by testing 100 nM, 200 nM, 400 nM and 800 nM primer in the PCR reaction in triplicate, with a constant probe concentration of 250 nM. Amplification curves were analysed visually and the lowest primer concentration that yielded an efficient amplification curve with the lowest cycle threshold (C_T) was selected to allow for multiplexed assays. Similarly, different probe concentrations in the PCR reaction (50 nM, 100 nM, 150 nM, 200 nM and 250 nM) were tested in triplicate, with a constant optimized primer concentration of 200 nM.

3.4 Nucleic acid extraction

Nucleic acid extraction was performed using the KingFisher™ Duo Prime Purification System. A standard extraction programme (MagMAX_Core_Duo_No_heat.bdz) was used. Purified nucleic acid was stored at -20°C until use if not used immediately.

3.4.1 Blood samples

Deoxyribonucleic acid (DNA) was extracted from blood samples using the MagMAX™ CORE Nucleic Acid Purification Kit (ThermoFisher Scientific, USA) and the simple workflow described by the manufacturer. Thirty microlitres bead/proteinase K (PK) mix was prepared by mixing 20 µl MagMAX CORE magnetic beads and 10 µl MagMAX™ CORE proteinase K and the mixture loaded into each well of row A of a Kingfisher deep-well 96 plate (ThermoFisher Scientific, USA). Whole blood samples (200 µl) were added into row A, followed by 350 µl MagMAX™ CORE Lysis Solution and 350 µl MagMAX™ CORE Binding Solution per well. MagMAX™ CORE Wash Solution 1 (500 µl) was added into each well of row B and MagMAX™ CORE Wash Solution 2 (500 µl) was added into each well of row C. The nucleic acid was eluted in 90 µl of MagMAX™ CORE Elution Buffer.

3.4.2 Whatman® Flinders Technology Associates (FTA) cards

There is no record of nucleic extraction from Whatman® FTA cards using the MagMAX™ CORE Nucleic Acid Purification Kit (ThermoFisher Scientific, USA), therefore, two standard MagMAX™ CORE Nucleic Acid Purification Kit protocols were run: the complex work flow and digestion work flow, were tested and compared. A Whatman® FTA card was placed on a sterile glass petri dish and approximately 10 mm^2 of the blood spot cut using sterile scalped blade and placed into 1.5 ml Eppendorf tubes.

3.4.2.1 Complex work flow

Two hundred microlitres 1X phosphate buffered saline (PBS) was added to the Whatman® FTA sample in the Eppendorf tube and vortexed for 3 min. Four hundred and fifty µl MagMAX CORE lysis solution was added to the tube and mixed thoroughly. Thirty microlitres bead/proteinase K (PK) mix (20 µl MagMAX CORE magnetic beads and 10 µl MagMAX CORE proteinase K per sample) was added into each well of row A of a Kingfisher deep-well 96 plate, together with 500 µl of sample solution. Five hundred µl of MagMAX™ CORE Wash Solutions 1 and 2 were added into rows B and C of the deep-well plate respectively. Ninety µl of elution buffer (MagMAX™ CORE Nucleic Acid Purification Kit) were added into each well of the elution strip.

3.4.2.2 Digestion work flow

Two hundred microlitres 1X PBS was added to the Whatman® FTA sample in Eppendorf tube and vortexed for 3 min. One hundred µl of PK solution (90 µl PK Buffer and 10 µl MagMAX™ CORE proteinase K) was added to each sample. The mixture was incubated for 30 min at 55°C and then 200 µl of the sample was added to 30 µl MagMAX CORE magnetic beads and 700 µl lysis/binding solution (350 µl MagMAX™ CORE Lysis Solution and 350 µl MagMAX™ CORE Binding Solution) in row A of a Kingfisher deep-well 96 plate. Five hundred µl of MagMAX™ CORE Wash Solutions 1 and 2 were added into Row B and C of the deep well plate respectively. Ninety µl of elution buffer were added into the elution strip.

3.5 Real-time polymerase chain reaction (PCR)

The components of the TaqMan® MGB real-time PCR assay consisted of 200 nM forward primer, 200 nM reverse primer, 250 nM probe, 10 µl 2x TaqMan® Fast Advanced Master Mix

(AmpliTaq® Fast DNA Polymerase, Uracil-N glycosylase (UNG), dNTPs (with dUTP), ROX™ dye, and optimized buffer) (ThermoFisher Scientific, USA), 2 µl sample template and distilled water to make up a reaction volume of 20 µl. This assay was performed on the StepOnePlus™ Real-Time PCR System (applied Bio systems) using the settings in Table 3.2.

Table 3.2 TaqMan® MGB real-time PCR assay conditions.

PCR steps	Number of cycles	Temperature (°C)	Duration
UNG incubation	1	50°C	2 min
Polymerase activation	1	95°C	20 sec
Denaturing	40	95°C	1 sec
Annealing/Extension	40	60°C	20 sec

3.6 Construction of a plasmid positive control

A plasmid control was produced from a diagnostic sample (RE16/076), which tested positive for *E. canis* by RLB hybridization assay and the TaqMan® MGB real-time PCR assay. A set of PCR primers (Table 3.3) targeting a region between nucleotides 41 and 330 of the 16S gene were designed in PrimerQuest® online (Integrated DNA Technologies, Inc. US) and synthesised by Integrated DNA Technologies (USA). Sequence JN622141 *E. canis* strain NGR clone 64 16S rRNA gene, partial sequence was used for numbering because it included the target region of the TaqMan® MGB real-time PCR assay.

Table 3.3 Plasmid construction primers designed to target nucleotides 41-330 bp of the *E. canis* 16S rRNA gene using JN622141 for numbering.

Name	Orientation	Start	Stop	Primer sequence (5'-3')	Length	TM (°C)	% GC
16S_F	Forward	41	62	TGCATGAGTCCAAGCCATAATG	22	59.6	45
16S_R	Reverse	302	330	TACGTTAGATTAGCTAGTTGGTGAG GTAA	29	58.3	38

The PCR reagents comprised of 12.5 µl 2x Phusion Flash High-Fidelity PCR master mix (Phusion DNA Polymerase, deoxynucleotides, Phusion HF Buffer and Phusion GC Buffer containing $MgCl_2$, Dimethyl sulfoxide) (ThermoFisher Scientific, USA), 20 µM forward primer 0.5 µl , 20 µM reverse primer 0.5 µl, 8.5 µl dH_2O and 2 µl template. Template amplification was performed on Gene Amp® PCR systems 2700 (Applied Biosystems) using the conditions listed in Table 3.4.

Table 3.4 PCR conditions to amplify *E. canis* 16S rRNA gene cloned region.

PCR steps	Number of cycles	Temperature (°C)	Duration
Initial denaturation	1	98°C	10 sec
Denaturation	30	98°C	1 sec
Annealing		55°C	5 sec
Extension		72°C	15 sec
Final Extension	1	72°C	1 min
		4°C	∞

Two microlitres of loading buffer (ThermoFisher Scientific, USA) was mixed with 5 µl PCR product and loaded on a 2% agarose gel prepared in 1x tris-acetate-ethylenediamine tetraacetic acid buffer (TAE) with ethidium bromide to visualize the DNA. The size of the band was determined with the aid of a 1 kb DNA ladder (ThermoFisher Scientific, USA). The PCR product was visualized with a ChemiDoc™ XRS+ System with Image Lab™ Software version 3.0 (Bio-Rad) Gel Doc imaging system.

The PCR product was purified using a High Pure PCR product purification kit (Roche, South Africa) and cloned into a pJET1.2/blunt cloning vector (ThermoFisher Scientific, USA). The competent high efficiency *Escherichia coli* JM109 cells (Promega, USA), were transformed using the vector following the manufacturer's instructions. imMedia™ Amp liquid broth (Invitrogen,USA) was used to grow the culture at 37°C for 1 h 30 min. Using standard procedure, the culture was plated on two imMedia™ Amp Blue (Invitrogen, USA) plates and incubated overnight at 37°C.

Colony PCR screening for the correct DNA insert was performed on the colonies using a reaction mixture comprised of 10 µl 2x Dream *Taq* buffer (ThermoFisher Scientific, USA), 0.4 µl 10 µM pJET1.2 forward sequencing primer, 0.4 µl 10 µM pJET1.2 reverse sequencing primer and 2 µl template, 9.2 µl dH_2O, using the conditions listed in Table 3.5. The PCR product was analyzed on a 2% agarose gel, as described earlier in 3.6.

Table 3.5 The *E. canis* (16S) colony PCR conditions.

PCR steps	Number of cycles	Temperature	Duration
Initial denaturation	1	95 °C	3 min
Denaturation	25	94 °C	30 sec
Annealing		60 °C	30 sec
Extension	1	72 °C	1 min
		4 °C	∞

To prepare glycerol stocks for each clone, selected colonies were grown overnight in imMedia™ Amp liquid broth (Invitrogen, USA) at 37°C in a shaking incubator. Five hundred microlitres of the culture was mixed gently with 500 µl 50% glycerol and stored at −80°C.

Plasmid extraction from overnight cultures was performed using a High Pure Plasmid Isolation Kit (Roche Diagnostics, Mannheim, Germany), by following the manufacturer's instructions. The eluted plasmid DNA concentration was determined by taking an average of multiple readings from BioTek™ PowerWave™ (Analytical and Diagnostic Products, South Africa) and Trinean Xpose (Anatech Instruments, South Africa) spectrophotometers. The copy number of the plasmid per µl was calculated using the formula: 6.022×10^{23} (copy number/mol) × concentration (g/µl) ÷ molar mass (g/mol) was used. A small volume of the recombinant plasmid (5 µl) was sent to Inqaba Biotec™ (Pretoria, RSA) for Sanger sequencing and the remaining plasmid stored at -20°C. Further analysis of the sequences was done using CLC Genomic Work bench 7 software (Qiagen bioinformatics). The National Centre for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast) BLAST search was used to identify the contigs.

3.7 Laboratory validation

3.7.1 Assay linear range and efficiency

A full blood cell count was performed on the blood from a greyhound dog that served as a blood donor for the OVAH and was tested on a regular basis for the presence of haemoparasites (Appendix 8.3).

A ten-fold dilution series (from 10^0 to 10^{-10}) of plasmid control DNA was prepared with TE buffer as the diluent. Ten µl of each plasmid DNA dilution was added to 190 µl of the greyhound blood to make a dilution series of plasmid spiked canine blood.

Nucleic acid extraction and TaqMan® MGB real-time PCR assay was carried out, as described in 3.4.1 and 3.5. Each dilution was tested in triplicate and the results used to generate a standard curve. The slope of the regression line was used to calculate the efficiency of the assay using the following equation: Efficiency (%) = $100 \times (10^{-1/\text{slope}} - 1)$.

3.7.2 Analytical sensitivity and variation

A starting dilution of 10^{-6} plasmid DNA was made to prepare a two-fold dilution series of blood spiked with $10^{1.43}$ to $10^{7.43}$ plasmid DNA, to cover the range from 100% to 0% pathogen detection. Five separate nucleic acid purifications were performed from each dilution. Five separate PCR runs were performed to test each purified dilution. The 95% limit of detection was calculated by probit analysis (SPSS Statistics v25, IBM Analytics,USA).

3.7.3 Analytical specificity

Assay specificity was evaluated by testing diagnostic n=4 samples submitted to the DVTD, University of Pretoria that had been found to be positive for other pathogens by the RLB hybridization assay (Table 3.6). Other tested samples included blood vaccines purchased from Onderstepoort Biological Products and in-house constructed plasmids.

Table 3.6 List of pathogens tested to determine the specificity of the TaqMan® MGB real-time PCR assay.

DNA sample	Reference
Anaplasma centrale	Frozen Anaplasmosis (Tick-Borne Gallsickness), Blood vaccine (Onderstepoort Biological Products) Reg. No.: G 1106 (Act 36/1947)
Babesia bigemina	Frozen African Redwater, Blood vaccine (Onderstepoort Biological Products) Reg. No.: G 1175 (Act 36/1947)
Babesia bovis	Frozen Asiatic Redwater, Blood vaccine (Onderstepoort Biological Products) Reg.No.: G 1106 (Act 36/1947)
Babesia caballi	Tissue culture obtained from Onderstepoort Veterinary Institute as a diagnostic sample
Babesia occultans	Tissue culture obtained from Onderstepoort Veterinary Institute as a diagnostic sample
Ehrlichia ruminantium	Heartwater - Infective Blood, Blood vaccine (Onderstepoort Biological Products) Reg. No.: G 0106 (Act 36/1947)
Theileria equi	RLB12/058
Theileria mutans	RE18/008
Theileria taurotragi	RE18/008
Theileria velifera	RE18/008
Babesia rossi	Plasmid constructed in-house
Babesia vogeli	Plasmid constructed in-house

3.7.4 Repeatability

Calculations of the intra-run and inter-run standard deviations (SD) and coefficient of variation (CV) were done with Microsoft Excel using results from 3.7.2. The total coefficient of variation (CV) was calculated by the formula: CV = total SD / (mean C_T value of all replicates).

3.8 Diagnostic validation

A total of 107 FTA samples from the DVTD biobank and 14 blood samples collected from OVAH and MAHC were tested with the TaqMan® MGB assay specific for *E. canis* detection. All results were compared with the RLB hybridization assay results.

Diagnostic sensitivity and specificity of the PCR assay were estimated in the absence of a gold standard assay, by using a two-test two-population Bayesian latent class model (Appendix 8.4) that allows for conditional dependence between tests (Branscum et al., 2005, Georgiadis et al., 2003). We assumed sensitivities and specificities were constant in the two populations (i.e. samples collected in South Africa and Namibia). Expert opinions were sought where no published references existed (Table 3.7).

Table 3.7 Prior values (mode and α and β-values of the beta distribution) used in a Bayesian latent class model for estimating the diagnostic sensitivity and specificity of a TaqMan® real-time PCR assay to detect *E. canis*. Pi1 - prevalence of *E. canis* in samples submitted to DVTD for testing of haemoparasites, Pi2 – prevalence of *E. canis* in samples from dogs with clinical signs of suspected ehrlichiosis.

	Mode	5/95th percentile	α-value	β-value	Reference
Sensitivity of RLB assay	0.85	0.70	23.90	5.04	-
Specificity of RLB assay	0.90	0.75	22.98	3.44	-
Sensitivity of PCR assay	0.90	0.20	1.94	1.10	Uniform prior
Specificity of PCR assay	0.90	0.20	1.94	1.10	Uniform prior
Pi1 (South Africa)	0.08	0.01	1.46	6.24	Prof A. Leisewitz, per.comm.
Pi2 (Namibia)	0.40	0.20	5.03	7.04	Prof B. Penzhorn, per. comm.

The model was run in OpenBUGS, version 3.2.3 rev 1012, a programme for Bayesian analysis of complex statistical models using Markov chain Monte Carlo (MCMC) techniques (Lunn et al., 2009, Gelfand and Smith, 1990). Two chains were used and initial values were generated by forward sampling from the prior distribution for each parameter. The first 10,000 iterations were discarded and the next 50,000 iterations used for posterior inferences. Model convergence was assessed by visual inspection of the trace plots.

Chapter 4
Results

4.1 TaqMan® MGB real-time PCR assay design

A total of 316 *E. canis* 16S rRNA sequences were downloaded and aligned. GenBank sequence NR_118741 was used as the reference sequence. Sequences AY394465, KF536734, EU376116, KF536738, KF536737 were removed due to the poor quality of the sequences. Sequences with two nucleotide indels were also removed, which left 166 sequences. Identical sequences were removed and this resulted in 64 unique sequences (Figure 4.1). The primers and probe were designed using Applied Biosystems Primer Express™ software following the recommended criteria, probe was 17 bp long, the melting temperature was 69°C, the percentage GC was 53% and there was no G remainder on the 5'-end since it has the capability to quench the fluorescence of the reporter dye.

The nucleotide variation within the *E. canis* 16S rRNA sequences downloaded from GenBank was plotted (Figure 4.2). The graph showed that amongst the sequences there is a high degree of variation in the 5' end in the region where the previously published assay was designed whereas the inverse was seen in the 3' end.

A BLAST of probes performed in GenBank® for homologous and heterologous species evaluation showed no non-specific binding, therefore, indicating high specificity of the TaqMan® MGB real-time PCR assay.

An alignment with different species in the *Ehrlichia*/*Anaplasma* genus was done to show the variation amongst the species in the *E. canis* probe region (Figure 4.3).

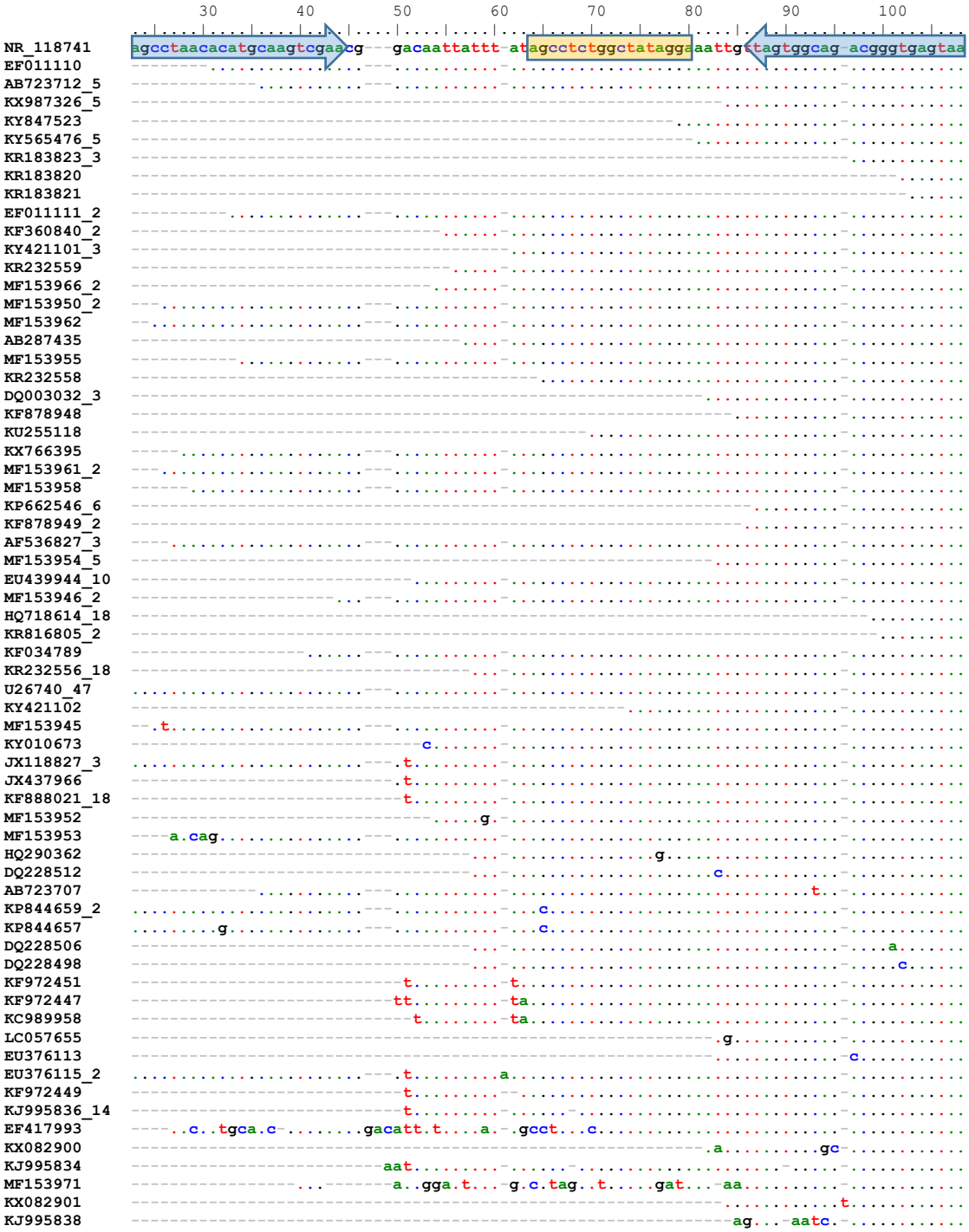

Figure 4.1 Variation of the 64 aligned *E. canis* sequences, the primers (represented by blue arrows) and probe region (represented by yellow rectangle) of a TaqMan® MGB assay to detect *E. canis*. Sequences are identified by the accession number followed by the number of sequence collapsed due to being identical to the first sequence. The dots represent identical nucleotide to the reference sequence and the dashed represent gaps.

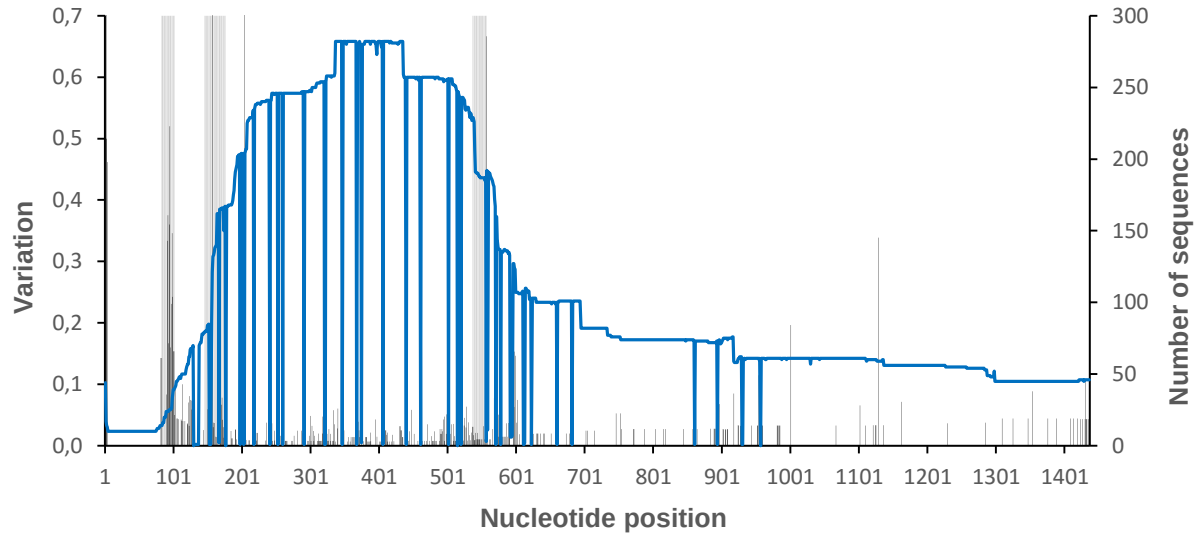

Figure 4.2 Graph plot representing the nucleotide position and variation of 316 *E. canis* 16S sequences downloaded from GenBank®. Nucleotide variation is represented by the black column height, the higher the column the greater the variation. No column represents a 100% conserved position. The blue line represents the number of sequences at each nucleotide position. The grey columns represent previously published nested PCR assay (Wen et al., 1997).

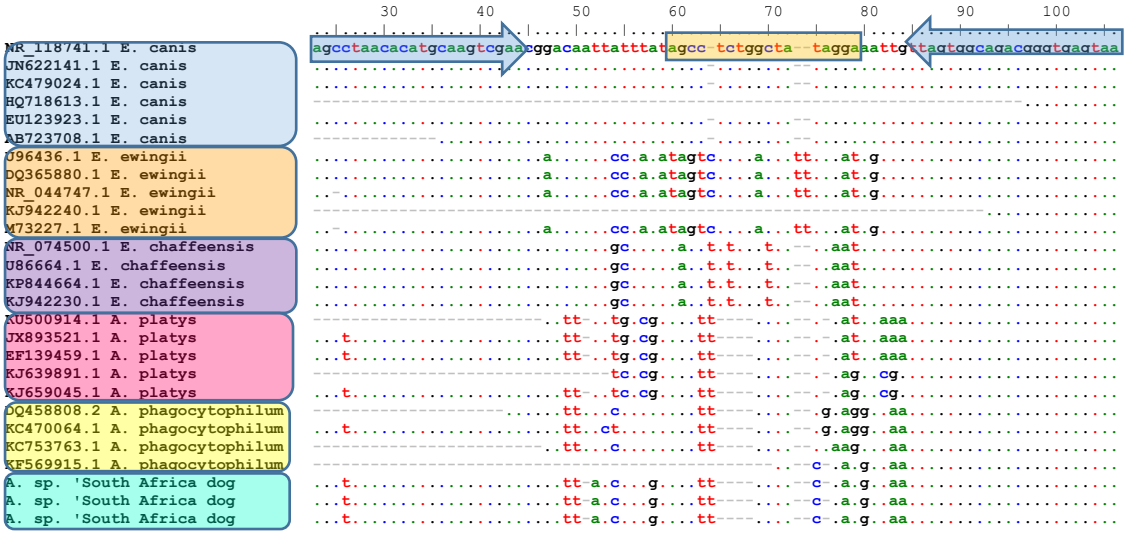

Figure 4.3 Variation of aligned *Ehrlichia* spp. sequences. The primers and probe of a TaqMan® MGB assay to detect *E. canis* are represented by blue arrows and a yellow rectangle, respectively. Sequences are identified by the accession number. The dots represent identical nucleotide to the reference sequence and the dashed represent gaps. NR_118741 was used as a reference sequence.

4.2 Primer and probe optimization

Four different primer concentrations were tested for optimization and the lowest, efficient primer concentration was determined to be 200 nM, as it yielded a low C_T value and a steep, efficient amplification curve reading (Figure 4.4).

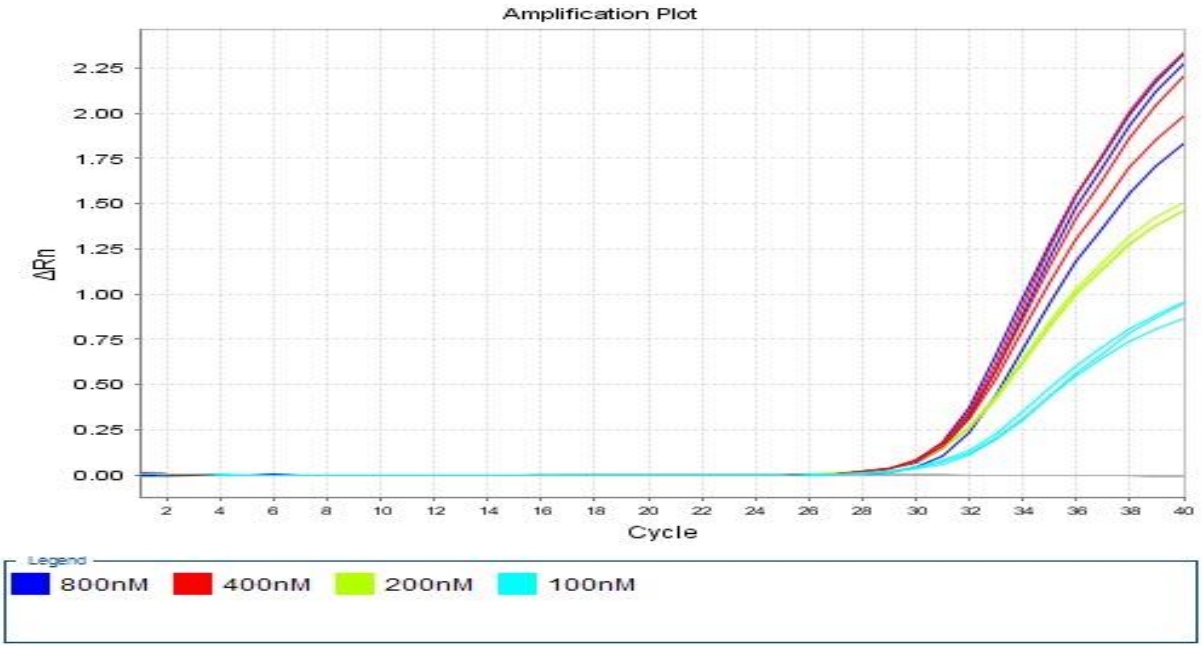

Figure 4.4 Primer concentration optimization curve of a TaqMan® MGB assay to detect *E. canis*.

The probe optimum concentration was determined by testing five different concentrations and the optimum probe concentration selected was 250 nM (Figure 4.5).

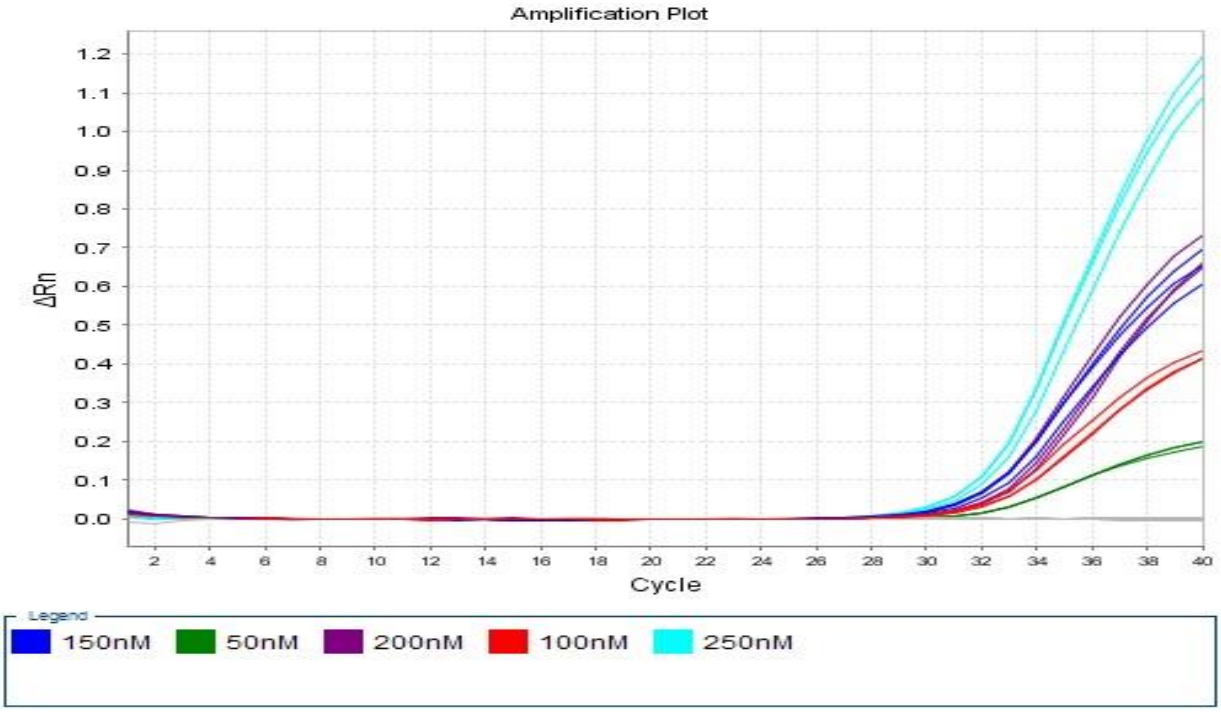

Figure 4.5 Probe concentration optimization curve of a TaqMan® MGB assay to detect *E. canis*.

4.3 Nucleic acid extraction from Whatman® FTA cards

Nucleic acid was purified from Whatman® FTA cards using two MagMAX™ CORE Nucleic Acid Purification Kit methods, the complex workflow and digestion workflow protocol. The results were compared, and the digestion workflow proved to be more appropriate for DNA extraction from Whatman® FTA cards, as the average difference was 3.00 C_T's lower using the digestion work flow, which equated to a 7.19-fold increase in sensitivity (using an efficiency of 93% for the assay) (Table 4.1).

Table 4.1 Comparison of real-time PCR results between MagMAX™ CORE Nucleic Acid Purification Kit complex and digestion extraction methods. C_T – cycle threshold.

Sample ID	Complex work flow (C_T-value)	Digestion work flow (C_T-value)	C_T-difference
P1	32.52	30.79	1.73
P2	31.41	30.75	0.66
P3	36.10	29.58	6.52
P4	34.93	31.82	3.11
P5	32.52	31.75	0.77
P6	32.41	30.76	1.65
P7	34.95	28.75	6.2
P8	Undetermined	29.68	-
P9	31.39	27.98	3.41

4.4 Plasmid positive control

Primers (Table 3.3) successfully amplified a 289 bp region which is inclusive of the TaqMan® MGB real-time PCR assay region of the *E. canis* 16S rRNA gene (Figure 4.6). The product was cloned into pJET vector (ThermoFisher Scientific, USA) and nine randomly picked colonies were analysed with pJET primers to check for the presence of the insert, visualized on a 2% agarose gel (Figure 4.7). Inqaba Biotec™ (Pretoria, RSA) Sanger sequencing results were analysed using CLC Genomic Work bench 7 software (Qiagen bioinformatics) and 100% identity with *E. canis* sequences was obtained.

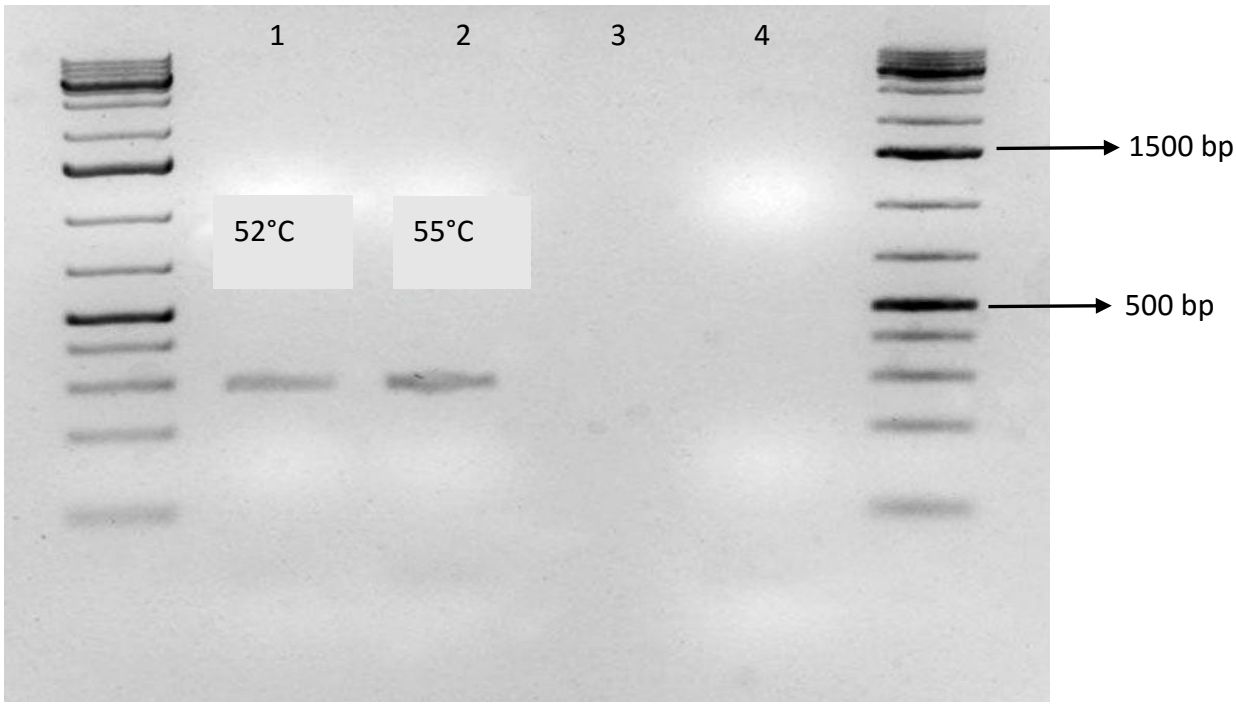

Figure 4.6 Visualisation of two 290 bp *E. canis* amplicons obtained at two different annealing temperatures (Lane 1 = 52°C and Lane 2 = 55°C), on a 2% agarose gel. The size of the bands were compared to a GeneRuler™ 1kb Plus DNA ladder (Thermo Scientific™, USA) in the outer wells.

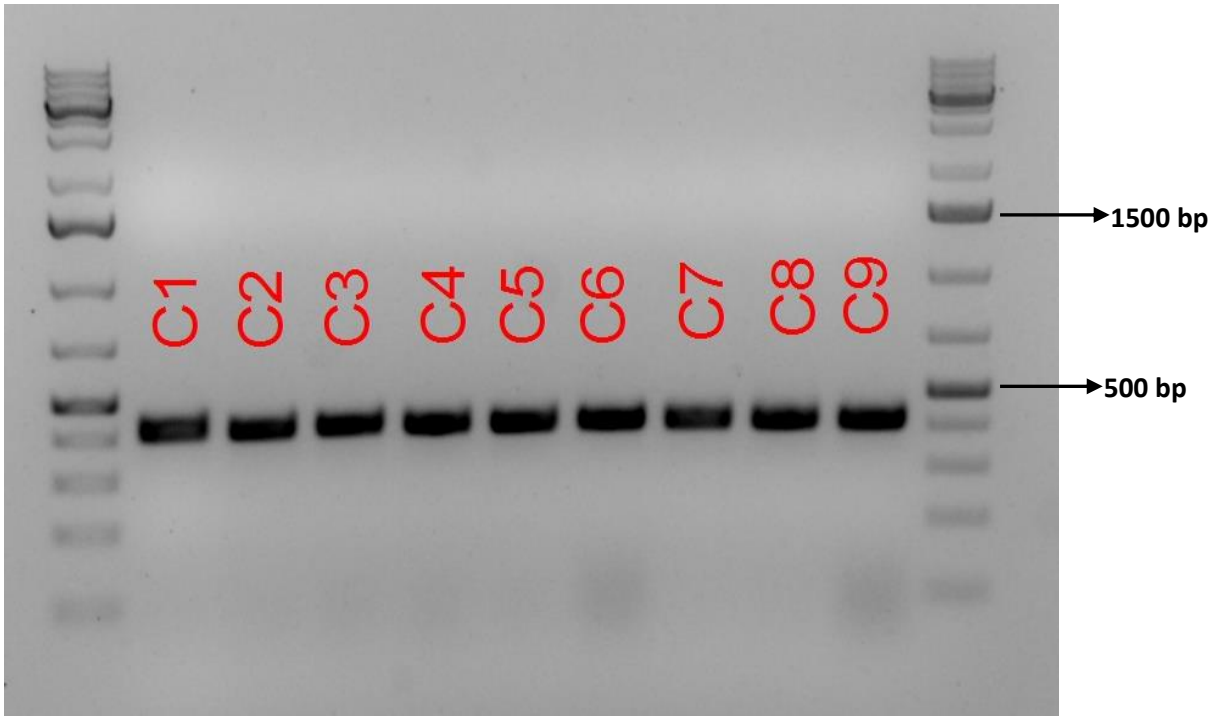

Figure 4.7 Amplicons from nine randomly picked colonies (C1 – C9), amplified using pJET primers run on a 2% agarose gel.

4.5 Laboratory validation

4.5.1 Assay linear range and efficiency

A standard curve was generated from canine blood spiked with *E. canis* plasmid to analyse the efficiency of the assay. The assay was linear between $10^{1.43}$ to $10^{7.43}$ plasmid copies/µl blood. The efficiency of the assay was 93%, while the correlation coefficient (R^2), a measure of how well the data fit the model and reflects the linearity of the standard curve was 0.9923 (Figure 4.8).

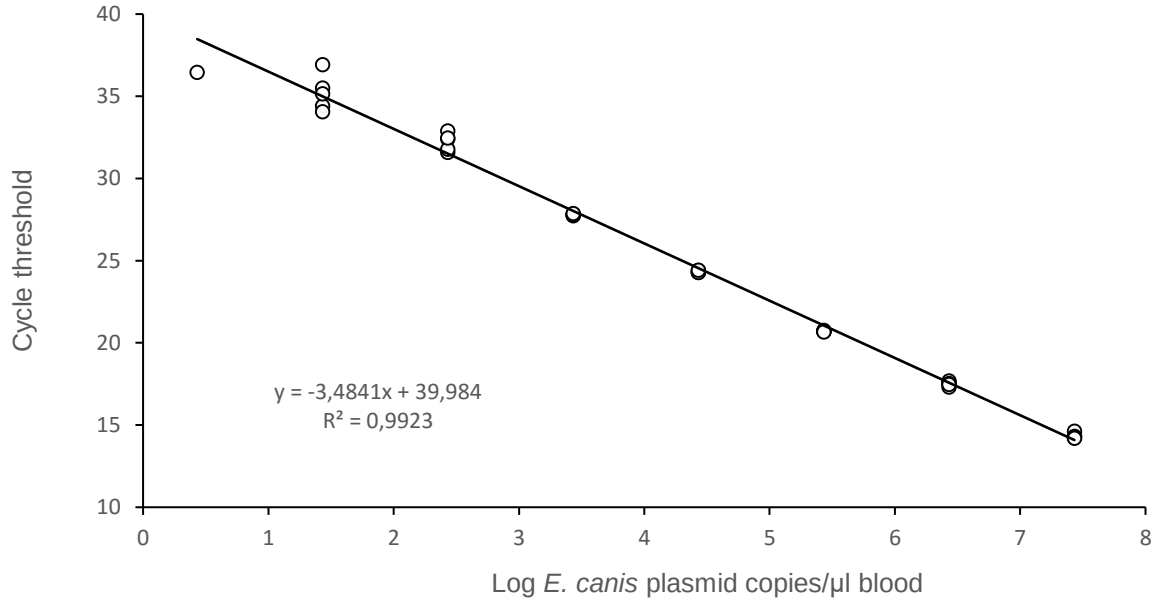

Figure 4.8 Standard curve displaying the linearity of the TaqMan® MGB real-time PCR assay from $10^{1.43}$ to $10^{7.43}$ plasmid copies/µl blood. The efficiency of the assay was 93%.

4.5.2 Analytical sensitivity

A two-fold dilution series of blood spiked with plasmid control DNA was made to cover the nonlinear range of the assay at the limit of detection. The 95% limit of detection (LOD) was 33.38 *E. canis* plasmid copies/µl of blood with a 95% confidence interval of 22.87 – 58.04 (Figure 4.9).

A cut-off C_T value of 37 was selected to categorise positive and negative samples. Above this value, samples were classified as negative and below this value as positive. This value equated to 7.18 *E. canis* plasmid copies /µl or a 55% LOD.

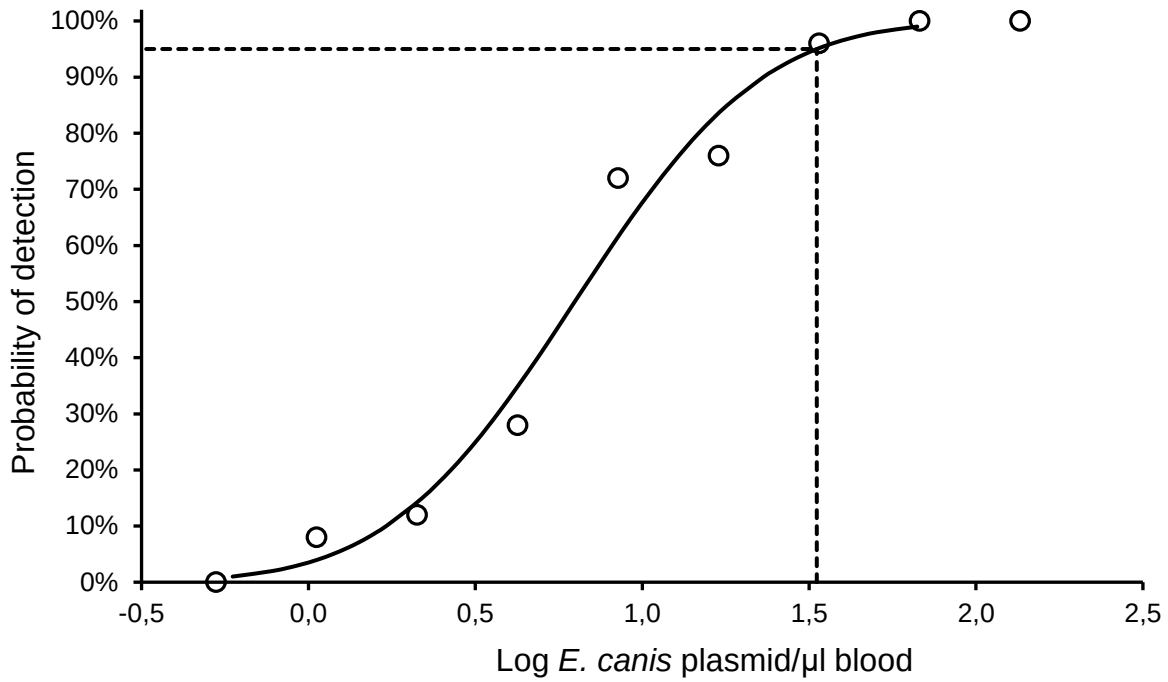

Figure 4.9 The 95% limit of detection (dotted line) of a TaqMan® MGB assay to detect *E. canis* 16S rRNA gene.

4.5.3 Analytical specificity

None of the pathogens tested (Table 3.6) cross-reacted with the TaqMan® MGB real-time PCR assay and only the *E. canis* positive control amplified (Figure 4.10).

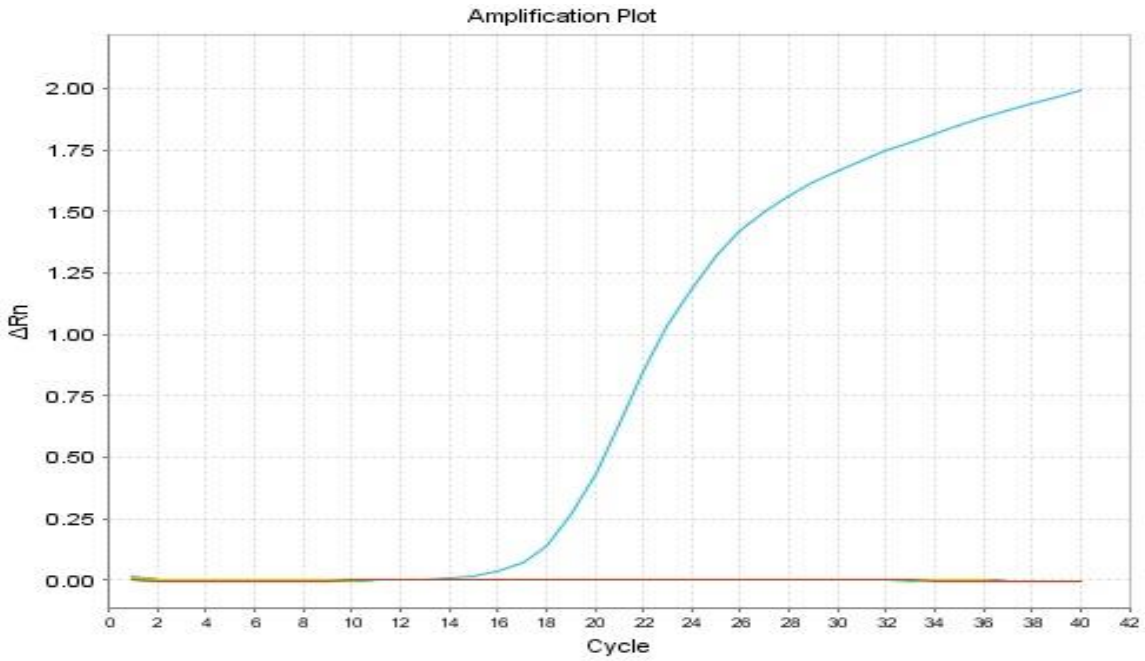

Figure 4.10 Specificity testing of a TaqMan® MGB assay to detect *E. canis* showed no amplification of a selection of pathogens, except for the *E. canis* control (blue line).

4.5.4 Repeatability

Intra- and inter-run standard deviation (SD) and coefficient of variation (CV) were used to evaluate the repeatability of the assay. The inter-run standard deviation (SD) ranged between 0.33 - 1.29 and the intra- run SD 0.04- 1.14. The coefficient of variation (CV) which indicate the variation between replicates and different runs ranged between 0.12 - 2.9 (Table 4.2).

Table 4.2 Variation of a TaqMan® MGB assay to detect *E. canis* plasmid of five replicates run in five runs. SD = standard deviation and CV = coefficient of variation.

Plasmid copies/µl blood	Inter-run SD	Intra-run SD	Total C_T Mean	Total SD	CV%
135.41	0.57	0.72	34.76	0.84	2.42
67.71	0.33	0.84	35.58	0.85	2.38
33.85	0.45	0.85	36.17	0.88	2.44
16.93	0.59	1.14	37.90	1.10	2.89
8.46	1.05	0.67	38.10	1.06	2.79
4.23	1.29	0.48	38.30	1.09	2.86
2.12	-	0.04	38.90	0.05	0.12
1.06	-	-	38.80	0.42	1.08

4.6 Diagnostic validation

The results for the two tests were in agreement (Table 4.3), with the exception of three samples. RE16/087 was positive (C_T of 32.97) with the TaqMan® MGB real-time PCR assay while on the RLB hybridization assay it was negative. RE16/103 was positive with (C_T of 35.92) when tested with the TaqMan® MGB real-time PCR assay and negative for *E. canis* on RLB hybridization assay however positive for *Babesia vogeli*, T/B catchall, B1 catchall.

Other samples that were collected at OVAH (five) and MACH (nine) were also tested with the TaqMan® MGB real-time PCR assay and two of the samples from OVAH were positive and three from the MACH were positive.

Table 4.3 Agreement/disagreement of the results of testing dog samples from South African and Namibia with the RLB hybridization assay and TaqMan® MGB assay specific for *E. canis* (PCR).

	Namibia samples		S. African samples	
	PCR+	PCR-	PCR+	PCR-
RLB+	9	0	31	0
RLB-	0	57	2	22

Using a Bayesian latent class model, the sensitivity of the TaqMan® MGB real-time PCR assay was 90%, which was slightly higher than the sensitivity of the RLB hybridization assay (87%). The specificity of the PCR and RLB hybridization assay were similar (92%) (Table 4.4).

Table 4.4 Estimates of the diagnostic sensitivity and specificity of the TaqMan® MGB real-time PCR assay and RLB hybridization assay.

Test parameter	Median	95% probability interval
Sensitivity of the PCR assay	0.9017	0.7869 - 0.9730
Specificity of the PCR assay	0.9254	0.8429 - 0.9798
Sensitivity of the RLB assay	0.8662	0.7620 - 0.9373
Specificity of the RLB assay	0.9249	0.8444 - 0.9768

4.7 Development of a Standard Operating Procedure (SOP)

A standard operating procedure was developed for use in the DVTD, UP (Appendix 8.5).

Chapter 5
Discussion

In this study we developed and validated a species-specific real-time PCR assay to detect *Ehrlichia canis* DNA in dogs. This was achieved by designing an *E. canis* TaqMan® MGB real-time PCR assay targeting the 16S rRNA gene with genus-specific primers and a probe specific to *E. canis*. The assay primers were designed to include all nucleotide variation within the target region and comprised a redundancy in the forward primer to allow for the detection of different species in the genus. The species considered when designing primers were *E. chaffeensis*, *E. ewingii*, *A. platys*, *A. phagocytophilum*, and *Anaplasma* sp. South Africa dog. Probes for the listed species were also designed but they were not tested in this study.

The 16S rRNA gene otherwise known as the 30S small subunit was chosen as the target gene for the development of the TaqMan® MGB real-time PCR assay. This gene is highly conserved, similar to other bacteria and is frequently used for classification of bacteria and development of group- or species-specific probes (Clarridge, 2004, Bottger, 1989). Diverse regions for species-specific probe design are very limited in the 16S rRNA gene, therefore, designing probes to distinguish species in a particular genus can be challenging. However, TaqMan® MGB probes allow shorter probe design, which is useful for shorter conserved regions identification in a variable region. TaqMan® MGB probes also have increased assay sensitivity due to lower background signal since the 3'-end has a non-fluorescent quencher-MGB attached to it (Kutyavin et al., 2000). Consequently, a TaqMan® MGB probe was designed in a conserved target sequence of the *E. canis* 16S rRNA gene.

There are a number of molecular assays that have been published targeting different genes, e.g. *p30* outer membrane proteins, citrate synthase (*gltA*) and heat-shock operon (*groEL*) gene to detect *E. canis* in canid blood (Ybañez et al., 2012, Stich et al., 2002). Thomson et al., (2018) established that the TaqMan® method was more specific for canine ehrlichiosis diagnosis when targeting the citrate synthase *gltA* in contrast to the PCRun® targeting the 16S rRNA gene.

Primers and probe designed for this study could successfully amplify and detect *E. canis* DNA in samples yielding good application curves with strong positive C_T values. Optimization of the primers and probe concentration yielded, 200 nM and 250 nM respectively which were

equivalent to what was obtained in a multiplex real-time PCR assay for canine haemoparasites *Babesia rossi* and *B. vogeli* (Troskie et al., 2019).

We successfully extracted DNA for the first time from Whatman® FTA cards using the digestive work and complex methods. The complex workflow proved to be more appropriate for Whatman® FTA extraction by yielding lower C_T values compared to those of the complex workflow.

In order to validate this assay, we simulated natural conditions by spiking blood from an *E. canis* negative donor dog (Appendix 8.3) with different concentrations of a plasmid DNA containing the target gene. There are numerous factors which can affect the efficiency of PCR amplification. These include magnesium and salt concentrations, reaction conditions, PCR target size and composition, primer sequences, and sample purity (Nolan and Bustin, 2013, Heid et al., 1996). The efficiency of the assay was measured as the slope of the semi-log regression line plot of C_T-value plotted against $\log_{10}$ of input nucleic acid. The efficiency was 93%, which was higher than 88% obtained in a multiplex assay for *E. canis* and *B. canis vogeli* (Peleg et al., 2010). The *E. canis* 16S rRNA TaqMan® MGB real-time PCR assay efficiency in this study was higher because this is a singleplex assay, with no primer-primer or probe-primer competition forming non-specific products. The 93% efficiency was within the acceptable range of 90% - 110% (Rogers-Broadway and Karteris, 2015).

The TaqMan® MGB real-time PCR assay was shown to be highly sensitive, by detecting 7.18 *E. canis* plasmid copies/µl which was more sensitive than the 12 parasites/µl obtained with a real-time multiplex PCR assays by Hojgaard et al., (2014).

The TaqMan® MGB real-time PCR assay described in this study proved to be specific for the target organism with no cross reactivity observed when tested against other haemoparasites.

The 95% limit of detection (LOD) was 33.38 *E. canis* plasmid copies/µl. A C_T value of 37 was selected as an adequate cut-off to categorise positive and negative samples, this value is similar to 38 C_T that was selected by Modarelli et al., (2019) as acceptable to detect potentially weak positive samples. Bhoora et al., (2018) reported a corresponding cut-off range of 35–37 for their multiplex assay.

A total of 121 field samples preserved on Whatman® FTA cards and blood samples were tested with both RLB hybridization assay and TaqMan® MGB real-time PCR assay and the

results were compared. Two false negative by RLB hybridization assay, sample RE16/075 and RE16/087 samples were obtained. This results could be due to poor DNA quality or DNA concentration lower than the limit of detection of the assay.

Analysis by Bayesian latent class model showed that the TaqMan® MGB real-time PCR assay is more sensitive and as specific as the RLB hybridization assay.

A standard operating procedure was produced and will be used by the Department of Veterinary Tropical Diseases as a guide to perform this test for diagnostic purposes (Appendix 8.5).

Real-time PCR eliminates electrophoresis analysis to visualize the PCR product because flourogenic probes permits the detection of specific amplification products and prevents non-specific amplification. This is advantageous in saving time and reagents. It also has an advantage of reducing potential PCR contamination in the laboratory since amplified products can be analysed and disposed of without opening the reaction tubes.

The shortcoming of TaqMan® MGB real-time PCR assay is that it uses a unique, more advanced and expensive thermocycler which is not available in most laboratories. The reagents are also more expensive than those of conventional PCR. It also has limited multiplexing capabilities due to the number of dyes available, nevertheless, it provides a convenient way of obtaining reliable results in a short time.

It is essential to detect ehrlichiosis disease early, as dogs who are not treated during the acute phase will enter into a chronic state of infection (Harrus and Waner, 2011). Since numerous important tick-borne pathogens like *B. rossi, B. vogeli* and *E. canis* can co-exist in the canine hosts causing severe clinical illness (Matjila et al., 2008). The availability of a fast, extremely sensitive, and specific test that can detect one or more pathogens, including co-infections, in a test sample will be of great value for timely diagnosis and treatment, cost reduction and labour related with singleplex reactions. Therefore, future studies can look at developing a multiplex molecular assay or a microplate bead-based multiplex immunoassay using Luminex xMAP technology that can detect up to 500 targets in a sample depending on the system design.

Chapter 6
Conclusion

The results from this study demonstrated that *E. canis* 16S rRNA TaqMan® real-time PCR assay is a rapid, sensitive and specific method for detecting *E. canis* DNA. The assay will be able to correctly and rapidly diagnose CME in dogs. This will allow for treatment to be administered in the early stages of the disease, speeding up the recovery time in affected dogs. Furthermore, it will limit the potential of toxicity due to shotgun treatment procedures, reduce the period of hospitalization and prevent the inappropriate use of antibiotics, thus minimising the potential for resistant strains to emerge. The test can also be useful for monitoring and controlling the spread of infections from ticks.

Chapter 7
References

ALVAREZ, M. J., VILA-ORTIZ, G. J., SALIBE, M. C., PODHAJCER, O. L. & PITOSSI, F. J. 2007. Model based analysis of real-time PCR data from DNA binding dye protocols. *BMC Bioinformatics,* 8**,** 85-94.

BATMAZ, H., NEVO, E., WANER, T., SENTURK, S., YILMAZ, Z. & HARRUS, S. 2001. Seroprevalence of *Ehrlichia canis* antibodies among dogs in Turkey. *Veterinary Record,* 148**,** 665-666.

BEKKER, C. P. J., DE VOS, S., TAOUFIK, A., SPARAGANO, O. A. E. & JONGEJAN, F. 2002. Simultaneous detection of *Anaplasma* and *Ehrlichia* species in ruminants and detection of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks by reverse line blot hybridization. *Veterinary Microbiology,* 89**,** 223-238.

BELL, C. A. & PATEL, R. 2005. A real-time combined polymerase chain reaction assay for the rapid detection and differentiation of *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis*, and *Ehrlichia ewingii*. *Diagnostic Microbiology and Infectious Disease,* 53**,** 301-306.

BERGGOETZ, M., SCHMID, M., STON, D., WYSS, V., CHEVILLON, C. & PRETORIUS, A. M. 2014. Protozoan and bacterial pathogens in tick salivary glands in wild and domestic animal environments in South Africa. *Ticks and Tick-borne Diseases,* 5**,** 176-185.

BHOORA, R. V., PIENAAR, R., CORNELIUS, F., JOSEMANS, A., MATTHEE, O., MARUMO, R., TROSKIE, C. & MANS, B. J. 2018. Multiplex hydrolysis-probe assay for the simultaneous detection of *Theileria equi* and *Babesia caballi* infections in equids. *Veterinary Parasitology,* 255**,** 61-68.

BOTTGER, E. C. 1989. Rapid determination of bacterial ribosomal RNA sequences by direct sequencing of enzymatically amplified DNA. *FEMS Microbiology Letters,* 53**,** 171-176.

BRANSCUM, A. J., GARDNER, I. A. & JOHNSON, W. O. 2005. Estimation of diagnostic-test sensitivity and specificity through Bayesian modeling. *Preventive Veterinary Medicine,* 68**,** 145-163.

BREITSCHWERDT, E. B., HEGARTY, B. C. & HANCOCK, S. I. 1998. Doxycycline Hyclate Treatment of Experimental Canine Ehrlichiosis Followed by Challenge Inoculation with Two *Ehrlichia canis* Strains. *Antimicrobial Agents and Chemotherapy,* 42**,** 362-368.

BREMER, W. G., SCHAEFER, J. J., WAGNER, E. R., EWING, S. A., RIKIHISA, Y., NEEDHAM, G. R., JITTAPALAPONG, S., MOORE, D. L. & STICH, R. W. 2005. Transstadial and intrastadial experimental transmission of *Ehrlichia canis* by male *Rhipicephalus sanguineus*. *Veterinary Parasitology,* 131**,** 95-105.

BUHLES, W. C., JR., HUXSOLL, D. L. & RISTIC, M. 1974. Tropical canine pancytopenia: Clinical, hematologic, and serologic response of dogs to *Ehrlichia canis* infection, tetracycline therapy, and challenge inoculation. *Journal of Infectious Diseases,* 130**,** 357-367.

CARDENAS, A. M., DOYLE, C. K., ZHANG, X., NETHERY, K., CORSTVET, R. E., WALKER, D. H. & MCBRIDE, J. W. 2007. Enzyme-linked immunosorbent assay with conserved immunoreactive glycoproteins gp36 and gp19 has enhanced sensitivity and provides species-specific immunodiagnosis of *Ehrlichia canis* infection. *Clinical and Vaccine Immunology,* 14**,** 123-128.

CARVALHO, L., ARMUA-FERNANDEZ, M. T., SOSA, N., FÉLIX, M. L. & VENZAL, J. M. 2017. *Anaplasma platys* in dogs from Uruguay. *Ticks and Tick-borne Diseases,* 8**,** 241-245.

CLARRIDGE, J. E., 3RD 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Reviews,* 17**,** 840-862.

COHN, L. A. 2003. Ehrlichiosis and related infections. *Veterinary Clinics of North America: Small Animal Practice,* 33**,** 863-884.

DAWSON, J. E., BIGGIE, K. L., WARNER, C. K., COOKSON, K., JENKINS, S., LEVINE, J. F. & OLSON, J. G. 1996. Polymerase chain reaction evidence of *Ehrlichia chaffeensis*, an etiologic agent of human ehrlichiosis, in dogs from southeast Virginia. *American Journal of Veterinary Research,* 57**,** 1175-1179.

DOYLE, C. K., LABRUNA, M. B., BREITSCHWERDT, E. B., TANG, Y.-W., CORSTVET, R. E., HEGARTY, B. C., BLOCH, K. C., LI, P., WALKER, D. H. & MCBRIDE, J. W. 2005. Detection of Medically Important *Ehrlichia* by Quantitative Multicolor TaqMan Real-Time Polymerase Chain Reaction of the dsb Gene. *The Journal of Molecular Diagnostics : JMD,* 7**,** 504-510.

DUMLER, J. S., BARBET, A. F., BEKKER, C. P., DASCH, G. A., PALMER, G. H., RAY, S. C., RIKIHISA, Y. & RURANGIRWA, F. R. 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*, descriptions of six 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.

EWING, S. A., ROBERSON, W. R., BUCKNER, R. G. & HAYAT, C. S. 1971. A new strain of *Ehrlichia canis*. *Journal of the American Veterinary Medical Association,* 159**,** 1771-1774.

FOURIE, J. J., HORAK, I., CRAFFORD, D., ERASMUS, H. L. & BOTHA, O. J. 2015. The efficacy of a generic doxycycline tablet in the treatment of canine monocytic ehrlichiosis. *Journal of the South African Veterinary Association,* 86**,** 1193-1202.

FOURIE, J. J., STANNECK, D., LUUS, H. G., BEUGNET, F., WIJNVELD, M. & JONGEJAN, F. 2013. Transmission of *Ehrlichia canis* by *Rhipicephalus sanguineus* ticks feeding on dogs and on artificial membranes. *Veterinary Parasitology,* 197**,** 595-603.

GAL, A., HARRUS, S., ARCOH, I., LAVY, E., AIZENBERG, I., MEKUZAS-YISASCHAR, Y. & BANETH, G. 2007. Coinfection with multiple tick-borne and intestinal parasites in a 6-week-old dog. *Canadian Veterinary Journal,* 48**,** 619–622.

GAL, A., LOEB, E., YISASCHAR-MEKUZAS, Y. & BANETH, G. 2008. Detection of *Ehrlichia canis* by PCR in different tissues obtained during necropsy from dogs surveyed for naturally occurring canine monocytic ehrlichiosis. *Journal of Veterinary Science,* 175**,** 212-217.

GAUNT, S. D., CORSTVET, R. E., BERRY, C. M. & BRENNAN, B. 1996. Isolation of *Ehrlichia canis* from dogs following subcutaneous inoculation. *Journal of Clinical Microbiology,* 34**,** 1429–1432.

GELFAND, A. E. & SMITH, A. F. M. 1990. Sampling-Based Approaches to Calculating Marginal Densities. *Journal of the American Statistical Association,* 85**,** 398-409.

GEORGIADIS, M. P., JOHNSON, W. O., GARDNER, I. A. & SINGH, R. 2003. Correlation-adjusted estimation of sensitivity and specificity of two diagnostic tests. *Journal of the Royal Statistical Society: Series C (Applied Statistics),* 52**,** 63-76.

GROVES, M. G., DENNIS, G. L., AMYX, H. L. & HUXSOLL, D. L. 1975. Transmission of *Ehrlichia canis* to dogs by ticks (*Rhipicephalus sanguineus*). *American Journal of Veterinary Research,* 36**,** 937-940.

GUBBELS, J. M., DE VOS, A. P., VAN DER WEIDE, M., VISERAS, J., SCHOULS, L. M., DE VRIES, E. & JONGEJAN, F. 1999. Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. *Journal of Clinical Microbiology,* 37**,** 1782-1789.

GUSA, A. A., BULLER, R. S., STORCH, G. A., HUYCKE, M. M., MACHADO, L. J., SLATER, L. N., STOCKHAM, S. L. & MASSUNG, R. F. 2001. Identification of a p28 Gene in *Ehrlichia ewingii*: Evaluation of Gene for Use as a Target for a Species-Specific PCR Diagnostic Assay. *Journal of Clinical Microbiology,* 39**,** 3871-3876.

HALL, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series,* 41**,** 95-98.

HARRUS, S. 2015. Perspectives on the pathogenesis and treatment of canine monocytic ehrlichiosis (*Ehrlichia canis*). *Journal of Veterinary Science,* 204**,** 239-240.

HARRUS, S., ALLEMAN, A. R., BARK, H., MAHAN, S. M. & WANER, T. 2002. Comparison of three enzyme-linked immunosorbant assays with the indirect immunofluorescent antibody test for the diagnosis of canine infection with *Ehrlichia canis*. *Veterinary Microbiology,* 86**,** 361-368.

HARRUS, S., AROCH, I., LAVY, E. & BARK, H. 1997a. Clinical manifestations of infectious canine cyclic thrombocytopenia. *Veterinary Record,* 141**,** 247-250.

HARRUS, S., KASS, P. H., KLEMENT, E. & WANER, T. 1997b. Canine monocytic ehrlichiosis: a retrospective study of 100 cases, and an epidemiological investigation of prognostic indicators for the disease. *Veterinary Record,* 141**,** 360-363.

HARRUS, S., KENNY, M., MIARA, L., AIZENBERG, I., WANER, T. & SHAW, S. 2004. Comparison of simultaneous splenic sample PCR with blood sample PCR for diagnosis and treatment of experimental *Ehrlichia canis* infection. *Antimicrobial Agents and Chemotherapy,* 48**,** 4488-4490.

HARRUS, S. & WANER, T. 2011. Diagnosis of canine monocytotropic ehrlichiosis (*Ehrlichia canis*): an overview. *Journal of Veterinary Science,* 187**,** 292-296.

HEGARTY, B. C., DE PAIVA DINIZ, P. P., BRADLEY, J. M., LORENTZEN, L. & BREITSCHWERDT, E. 2009. Clinical relevance of annual screening using a commercial enzyme-linked immunosorbent assay (SNAP 3Dx) for canine ehrlichiosis. *Journal of the American Animal Hospital Association* 45**,** 118-124.

HEGARTY, B. C., LEVY, M. G., GAGER, R. F. & BREITSCHWERDT, E. B. 1997. Immunoblot analysis of the immunoglobulin G response to *Ehrlichia canis* in dogs: an international survey. *Journal of Veterinary Diagnostic Investigation* 9**,** 32-38.

HEID, C. A., STEVENS, J., LIVAK, K. J. & WILLIAMS, P. M. 1996. Real time quantitative PCR. *Genome Research,* 6**,** 986-994.

HIGUCHI, R., FOCKLER, C., DOLLINGER, G. & WATSON, R. 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology (N Y),* 11**,** 1026-1030.

HILDEBRANDT, P. K., CONROY, J. D., MCKEE, A. E., NYINDO, M. B. & HUXSOLL, D. L. 1973. Ultrastructure of *Ehrlichia canis*. *Infection and Immunity,* 7**,** 265-271.

HINRICHSEN, V. L., WHITWORTH, U. G., BREITSCHWERDT, E. B., HEGARTY, B. C. & MATHER, T. N. 2001. Assessing the association between the geographic distribution of deer ticks and seropositivity rates to various tick-transmitted disease organisms in dogs. *Journal of the American Veterinary Medical Association,* 218**,** 1092-1097.

HOJGAARD, A., LUKACIK, G. & PIESMAN, J. 2014. Detection of *Borrelia burgdorferi, Anaplasma phagocytophilum* and *Babesia microti*, with two different multiplex PCR assays. *Ticks and Tick-borne Diseases,* 5**,** 349-351.

HORAK, I. G., FOURIE, L. J., HEYNE, H., WALKER, J. B. & NEEDHAM, G. R. 2002. *Ixodid* Ticks Feeding on Humans in South Africa: with Notes on Preferred Hosts, Geographic Distribution, Seasonal Occurrence and Transmission of Pathogens. *Experimental & Applied Acarology,* 27**,** 113-136.

INOKUMA, H., OYAMADA, M., KELLY, P. J., JACOBSON, L. A., FOURNIER, P.-E., ITAMOTO, K., OKUDA, M. & BROUQUI, P. 2005. Molecular detection of a new *Anaplasma* species closely related to *Anaplasma phagocytophilum* in canine blood from South Africa. *Journal of Clinical Microbiology,* 43**,** 2934-2937.

IQBAL, Z., CHAICHANASIRIWITHAYA, W. & RIKIHISA, Y. 1994. Comparison of PCR with other tests for early diagnosis of canine ehrlichiosis. *Journal of Clinical Microbiology,* 32**,** 1658-1662.

IQBAL, Z. & RIKIHISA, Y. 1994. Reisolation of *Ehrlichia canis* from blood and tissues of dogs after doxycycline treatment. *Journal of Clinical Microbiology,* 32**,** 1644-1649.

IWERIEBOR, B. C., MMBAGA, E. J., ADEGBORIOYE, A., IGWARAN, A., OBI, L. C. & OKOH, A. I. 2017. Genetic profiling for *Anaplasma* and *Ehrlichia* species in ticks collected in the Eastern Cape Province of South Africa. *BMC Microbiology,* 17**,** 45-52.

JACOBS, P. A. H., FOURIE, L. J., KOK, D. J. & HORAK, I. G. 2001. Diversity, seasonality and sites of attachment of adult *Ixodid* ticks on dogs in the central region of the Free State Province, South Africa. *Onderstepoort Journal of Veterinary Research,* 68**,** 281-290.

JOHNSON, E. M., EWING, S. A., BARKER, R. W., FOX, J. C., CROW, D. W. & KOCAN, K. M. 1998. Experimental transmission of *Ehrlichia canis* (Rickettsiales: Ehrlichieae) by *Dermacentor variabilis* (Acari: Ixodidae). *Veterinary Parasitology,* 74**,** 277-288.

KATOH, K., ROZEWICKI, J. & YAMADA, K. D. 2017. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Briefings in Bioinformatics***,** 1160-1166.

KIM, C. M., YI, Y. H., YU, D. H., LEE, M. J., CHO, M. R. & DESAI, A. R. 2006. Tick-borne rickettsial pathogens in ticks and small mammals in Korea. *Applied and Environmental Microbiology,* 72**,** 5766–5776.

KORDICK, S. K., BREITSCHWERDT, E. B., HEGARTY, B. C., SOUTHWICK, K. L., COLITZ, C. M., HANCOCK, S. I., BRADLEY, J. M., RUMBOUGH, R., MCPHERSON, J. T. & MACCORMACK, J. N. 1999. Coinfection with multiple tick-borne pathogens in a Walker Hound kennel in North Carolina. *Journal of Clinical Microbiology,* 37**,** 2631-2638.

KUTYAVIN, I. V., AFONINA, I. A., MILLS, A., GORN, V. V., LUKHTANOV, E. A., BELOUSOV, E. S., SINGER, M. J., WALBURGER, D. K., LOKHOV, S. G., GALL, A. A., DEMPCY, R., REED, M. W., MEYER, R. B. & HEDGPETH, J. 2000. 3'-minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. *Nucleic Acids Research,* 28**,** 655-661.

LITTLE, S. E. 2010. Ehrlichiosis and anaplasmosis in dogs and cats. *Veterinary Clinics of North America: Small Animal Practice,* 40**,** 1121-1140.

LUNN, D., SPIEGELHALTER, D., THOMAS, A. & BEST, N. 2009. The BUGS project: Evolution, critique and future directions. *Statistics in Medicine,* 28**,** 3049-3067.

MACKAY, I. M. 2004. Real-time PCR in the microbiology laboratory. *Clinical Microbiology and Infection,* 10**,** 190-212.

MAEDA, K., MARKOWITZ, N., HAWLEY, R. C., RISTIC, M., COX, D. & MCDADE, J. E. 1987. Human infection with *Ehrlichia canis*, a leukocytic rickettsia. *New England Journal of Medicine,* 316**,** 853-856.

MATJILA, P. T., LEISEWITZ, A. L., JONGEJAN, F. & PENZHORN, B. L. 2008. Molecular detection of tick-borne protozoal and ehrlichial infections in domestic dogs in South Africa. *Veterinary Parasitology,* 155**,** 152-157.

MATTHEWMAN, L. A., KELLY, P. J., BOBADE, P. A., TAGWIRA, M., MASON, P. R., MAJOK, A., BROUQUI, P. & RAOULT, D. 1993a. Infections with *Babesia canis* and *Ehrlichia canis* in dogs in Zimbabwe. *Veterinary Record,* 133**,** 344-346.

MATTHEWMAN, L. A., KELLY, P. J., MAHAN, S. M., SEMU, D., TAGWIRA, M., BOBADE, P. A., BROUQUI, P., MASON, P. R. & RAOULT, D. 1993b. Western blot and indirect fluorescent antibody testing for antibodies reactive with *Ehrlichia canis* in sera from apparently healthy dogs in Zimbabwe. *Journal of the South African Veterinary Association,* 64**,** 111-115.

MCBRIDE, J. W., CORSTVET, R. E., BREITSCHWERDT, E. B. & WALKER, D. H. 2001. Immunodiagnosis of *Ehrlichia canis* infection with recombinant proteins. *Journal of Clinical Microbiology,* 39**,** 315-322.

MCQUISTON, J. H., CHILDS, J. E., CHAMBERLAND, M. E. & TABOR, E. 2000. Transmission of tick-borne agents of disease by blood transfusion: a review of known and potential risks in the United States. *Transfusion,* 40**,** 274-284.

MODARELLI, J. J., FERRO, P. J., DE LEON, A. A. P. & ESTEVE-GASENT, M. D. 2019. TickPath Layerplex: adaptation of a real-time PCR methodology for the simultaneous detection and molecular surveillance of tick-borne pathogens. *Scientific Reports,* 9**,** 6950-6961.

MTSHALI, K., NAKAO, R., SUGIMOTO, C. & THEKISOE, O. 2017. Occurrence of *Coxiella burnetii*, *Ehrlichia canis*, Rickettsia species and *Anaplasma phagocytophilum*-like bacterium in ticks collected from dogs and cats in South Africa. *Journal of the South African Veterinary Association,* 88**,** 1-6.

MURPHY, G. L., EWING, S. A., WHITWORTH, L. C., FOX, J. C. & KOCAN, A. A. 1998. A molecular and serologic survey of *Ehrlichia canis*, *E. chaffeensis*, and *E. ewingii* in dogs and ticks from Oklahoma. *Veterinary Parasitology,* 79**,** 325-339.

MYLONAKIS, M. E., HARRUS, S. & BREITSCHWERDT, E. B. 2019. An update on the treatment of canine monocytic ehrlichiosis (*Ehrlichia canis*). *The Veterinary Journal,* 246**,** 45-53.

MYLONAKIS, M. E., KOUTINAS, A. F., BREITSCHWERDT, E. B., HEGARTY, B. C., BILLINIS, C. D., LEONTIDES, L. S. & KONTOS, V. S. 2004. Chronic canine ehrlichiosis (Ehrlichia canis): a

retrospective study of 19 natural cases. *Journal of the American Animal Hospital Association (JAAHA),* 40**,** 174-184.

MYLONAKIS, M. E. & THEODOROU, K. N. 2017. Canine Monocytic Ehrlichiosis: An Update on Diagnosis and Treatment. *Acta Veterinaria,* 67**,** 299-317.

NAZARI, M., LIM, S. Y., WATANABE, M., SHARMA, R. S., CHENG, N. A. & WATANABE, M. 2013. Molecular detection of *Ehrlichia canis* in dogs in Malaysia. *PLOS Neglected Tropical Diseases,* 7**,** e1982.

NDIP, L. M., NDIP, R. N., ESEMU, S. N., WALKER, D. H. & MCBRIDE, J. W. 2010. Predominance of *Ehrlichia chaffeensis* in *Rhipicephalus sanguineus* ticks from a kennel-confined dogs in Limbe, Cameroon. *Experimental and Applied Acarology,* 50**,** 163-168.

NEER, T. M., BREITSCHWERDT, E. B., GREENE, R. T. & LAPPIN, M. R. 2002. Consensus statement on ehrlichial disease of small animals from the infectious disease study group of the ACVIM. American College of Veterinary Internal Medicine. *Journal of Veterinary Internal Medicine,* 16**,** 309-315.

NEER, T. M. & HARRUS, S. 2006. *Canine monocytotropic ehrlichiosis and neorickettsiosis (E. canis, E. chaffeensis, E. ruminantium, N. sennetsu, and N. risticii infections)*.

NOLAN, T. & BUSTIN, S. A. 2013. *PCR technology: current innovations*, CRC press.

NYINDO, M., HUXSOLL, D. L., RISTIC, M., KAKOMA, I., BROWN, J. L., CARSON, C. A. & STEPHENSON, E. H. 1980. Cell-mediated and humoral immune responses of German Shepherd Dogs and Beagles to experimental infection with *Ehrlichia canis*. *American Journal of Veterinary Research,* 41**,** 250-254.

OGDEN, N. H., CASEY, A. N. J., WOLDEHIWET, Z. & FRENCH, N. P. 2003. Transmission of *Anaplasma phagocytophilum* to *Ixodes ricinus* ticks from sheep in the acute and post-acute phases of infection. *Infection and immunity,* 71**,** 2071-2078.

OIE-TERRESTRIAL-MANUAL. 2009. *OIE Terrestrial Manual* [Online]. [Accessed].

PAROLA, P., INOKUMA, H., CAMICAS, J. L., BROUQUI, P. & RAOULT, D. 2001. Detection and identification of spotted fever group *Rickettsiae* and *Ehrlichiae* in African ticks. *Emerging Infectious Diseases,* 7**,** 1014-1017.

PELEG, O., BANETH, G., EYAL, O., INBAR, J. & HARRUS, S. 2010. Multiplex real-time qPCR for the detection of *Ehrlichia canis* and *Babesia canis vogeli*. *Veterinary Parasitology,* 173**,** 292-299.

PEREZ, M., RIKIHISA, Y. & WEN, B. 1996. *Ehrlichia canis*-like agent isolated from a man in Venezuela: antigenic and genetic characterization. *Journal of Clinical Microbiology,* 34**,** 2133-2139.

PRETORIUS, A. & KELLY, P. 1998. Serological survey for antibodies reactive with *Ehrlichia canis* and *E. chaffeensis* in dogs from the Bloemfontein area, South Africa. *Journal of the South African Veterinary Association,* 69**,** 126-128.

RAUTENBACH, G., BOOMKER, J. & DE VILLIERS, I. 1991. A descriptive study of the canine population in a rural town in southern Africa. *Journal of the South African Veterinary Association,* 62**,** 158-162.

RAUTENBACH, Y., SCHOEMAN, J. & GODDARD, A. 2018. Prevalence of canine *Babesia* and *Ehrlichia* co-infection and the predictive value of haematology. *Onderstepoort Journal of Veterinary Research,* 85**,** e1-e5.

RICHTER, P. J., JR., KIMSEY, R. B., MADIGAN, J. E., BARLOUGH, J. E., DUMLER, J. S. & BROOKS, D. L. 1996. *Ixodes pacificus* (Acari: Ixodidae) as a vector of *Ehrlichia equi* (Rickettsiales: Ehrlichieae). *Journal of Medical Entomology,* 33**,** 1-5.

RIKIHISA, Y. 1991. The tribe *Ehrlichieae* and ehrlichial diseases. *Clinical Microbiology Reviews,* 4**,** 286-308.

RISTIC, M., HUXSOLL, D. L., TACHIBANA, N. & RAPMUND, G. 1981. Evidence of a serologic relationship between *Ehrlichia canis* and *Rickettsia sennetsu*. *American Society of Tropical Medicine and Hygiene (ASTMH)* 30**,** 1324-1328.

RISTIC, M., HUXSOLL, D. L., WEISIGER, R. M., HILDEBRANDT, P. K. & NYINDO, M. 1972. Serological diagnosis of tropical canine pancytopenia by indirect immunofluorescence. *Infection and Immunity,* 6**,** 226-231.

ROGERS-BROADWAY, K.-R. & KARTERIS, E. 2015. Amplification efficiency and thermal stability of qPCR instrumentation: Current landscape and future perspectives. *Experimental and Therapeutic Medicine,* 10**,** 1261-1264.

SAINZ, A., ROURA, X., MIRO, G., ESTRADA-PENA, A., KOHN, B., HARRUS, S. & SOLANO-GALLEGO, L. 2015. Guideline for veterinary practitioners on canine ehrlichiosis and anaplasmosis in Europe. *Parasites & Vectors,* 8**,** 75-95.

SCHOULS, L. M., VAN DE POL, I., RIJPKEMA, S. G. & SCHOT, C. S. 1999. Detection and identification of *Ehrlichia*, *Borrelia burgdorferi* sensu lato, and *Bartonella* species in Dutch *Ixodes ricinus* ticks. *Journal of Clinical Microbiology,* 37**,** 2215-2222.

SIRIGIREDDY, K. R. & GANTA, R. R. 2005. Multiplex detection of *Ehrlichia* and *Anaplasma* species pathogens in peripheral blood by real-time reverse transcriptase-polymerase chain reaction. *Journal of Molecular Diagnostics,* 7**,** 308-316.

SMITH, R. D., SELLS, D. M., STEPHENSON, E. H., RISTIC, M. R. & HUXSOLL, D. L. 1976. Development of *Ehrlichia canis*, causative agent of canine ehrlichiosis, in the tick *Rhipicephalus sanguineus* and its differentiation from a symbiotic *Rickettsia*. *American Journal of Veterinary Research,* 37**,** 119-126.

STICH, R. W., RIKIHISA, Y., EWING, S., NEEDHAM, G. R., GROVER, D. L. & JITTAPALAPONG, S. 2002. Detection of *Ehrlichia canis* in canine carrier blood and in individual experimentally infected ticks with a p30-based PCR assay. *Journal of Clinical Microbiology,* 40**,** 540-546.

STICH, R. W., SCHAEFER, J. J., BREMER, W. G., NEEDHAM, G. R. & JITTAPALAPONG, S. 2008. Host surveys, *ixodid* tick biology and transmission scenarios as related to the tick-borne pathogen, *Ehrlichia canis*. *Veterinary Parasitology,* 158**,** 256-273.

SYKES, J. E. 2013. *Canine and feline infectious diseases*.

SYKES, J. E. 2014. Chapter 28 - Ehrlichiosis. *In:* SYKES, J. E. (ed.) *Canine and Feline Infectious Diseases.* Saint Louis: W.B. Saunders.

THOMSON, K., YAARAN, T., BELSHAW, A., CURSON, L., TISI, L., MAURICE, S. & KIDDLE, G. 2018. A new TaqMan method for the reliable diagnosis of *Ehrlichia* spp. in canine whole blood. *Parasites & vectors,* 11**,** 350-350.

TICHOPAD, A., DILGER, M., SCHWARZ, G. & PFAFFL, M. W. 2003. Standardized determination of real-time PCR efficiency from a single reaction set-up. *Nucleic Acids Research,* 31**,** e122.

TROSKIE, M., DE VILLIERS, L., LEISEWITZ, A., OOSTHUIZEN, M. C. & QUAN, M. 2019. Development and validation of a multiplex, real-time PCR assay for *Babesia rossi* and *Babesia vogeli*. *Ticks and Tick-borne Diseases,* 10**,** 421-432.

UNVER, A., PEREZ, M., ORELLANA, N., HUANG, H. & RIKIHISA, Y. 2001. Molecular and antigenic comparison of *Ehrlichia canis* isolates from dogs, ticks, and a human in Venezuela. *Journal of Clinical Microbiology,* 39**,** 2788-2793.

VENTOLA, C. L. 2015. The antibiotic resistance crisis: part 1: causes and threats. *P & T : A Peer-Reviewed Journal for Formulary Management,* 40**,** 277-283.

WALKER, D. H. & DUMLER, J. S. 1996. Emergence of the ehrlichioses as human health problems. *Emerging Infectious Diseases,* 2**,** 18-29.

WANER, T., HARRUS, S., JONGEJAN, F., BARK, H., KEYSARY, A. & CORNELISSEN, A. W. 2001. Significance of serological testing for ehrlichial diseases in dogs with special emphasis on the diagnosis of canine monocytic ehrlichiosis caused by *Ehrlichia canis*. *Veterinary Parasitology,* 95**,** 1-15.

WANER, T., STRENGER, C. & KEYSARY, A. 2000. Comparison of a clinic-based ELISA test kit with the immunofluorescence test for the assay of *Ehrlichia canis* antibodies in dogs. *Journal of Veterinary Diagnostic Investigation (JVDI),* 12**,** 240-244.

WANER, T., STRENGER, C., KEYSARY, A. & HARRUS, S. 1998. Kinetics of serologic cross-reactions between *Ehrlichia canis* and the *Ehrlichia phagocytophila* genogroups in experimental *E. canis* infection in dogs. *Veterinary Immunology and Immunopathology,* 66**,** 237-243.

WEN, B., RIKIHISA, Y., MOTT, J. M., GREENE, R., KIM, H. Y., ZHI, N., COUTO, G. C., UNVER, A. & BARTSCH, R. 1997. Comparison of nested PCR with immunofluorescent-antibody assay for detection of *Ehrlichia canis* infection in dogs treated with doxycycline. *Journal of Clinical Microbiology,* 35**,** 1852-1855.

WILLIAMS, B. M., BERENTSEN, A., SHOCK, B. C., TEIXIERA, M., DUNBAR, M. R., BECKER, M. S. & YABSLEY, M. J. 2014. Prevalence and diversity of *Babesia*, *Hepatozoon*, *Ehrlichia*, and *Bartonella* in wild and domestic carnivores from Zambia, Africa. *Parasitology Research,* 113**,** 911-918.

WONG, M. L. & MEDRANO, J. F. 2005. Real-time PCR for mRNA quantitation. *BioTechniques,* 39**,** 75-85.

XIA, X. 2018. DAMBE7: New and Improved Tools for Data Analysis in Molecular Biology and Evolution. *Molecular Biology and Evolution,* 35**,** 1550-1552.

YABSLEY, M. J., MCKIBBEN, J., MACPHERSON, C. N., CATTAN, P. F., CHERRY, N. A., HEGARTY, B. C., BREITSCHWERDT, E. B., O'CONNOR, T., CHANDRASHEKAR, R., PATERSON, T., PEREA, M. L., BALL, G., FRIESEN, S., GOEDDE, J., HENDERSON, B. & SYLVESTER, W. 2008. Prevalence of *Ehrlichia canis*, *Anaplasma platys*, *Babesia canis vogeli*, *Hepatozoon canis, Bartonella vinsonii berkhoffii*, and *Rickettsia* spp. in dogs from Grenada. *Veterinary Parasitology,* 151**,** 279-285.

YBAÑEZ, A. P., PEREZ, Z. O., GABOTERO, S. R., YANDUG, R. T., KOTARO, M. & INOKUMA, H. 2012. First molecular detection of *Ehrlichia canis* and *Anaplasma platys* in ticks from Dogs in Cebu Philippines. *Ticks and Tick-borne Diseases,* 3**,** 288-293.

ZHANG, Q., WANG, J., DENG, F., YAN, Z., XIA, Y., WANG, Z., YE, J., DENG, Y., ZHANG, Z., QIAO, M., LI, R., DENDULURI, S. K., WEI, Q., ZHAO, L., LU, S., WANG, X., TANG, S., LIU, H., LUU, H. H., HAYDON, R. C., HE, T.-C. & JIANG, L. 2015. TqPCR: A Touchdown qPCR Assay with Significantly Improved Detection Sensitivity and Amplification Efficiency of SYBR Green qPCR. *PLOS ONE,* 10**,** e0132666.

Chapter 8

Appendix

8.1 Individual results of field samples

Sample ID	RLB result	Real-time PCR result	Sample origin
RE16/110	Positive	24.75	Namibia
RE16/084	Positive	24.94	Namibia
RE16/076	Positive	25.53	Namibia
RE16/108	Positive	26.70	Namibia
RE16/117	Positive	27.35	Namibia
RE16/113	Positive	27.40	Namibia
RE16/079	Positive	27.76	Namibia
RE16/098	Positive	27.82	Namibia
RE16/091	Positive	27.85	Namibia
RE16/073	Positive	27.98	Namibia
RE16/081	Positive	28.01	Namibia
RE16/088	Positive	28.47	Namibia
RE16/112	Positive	28.52	Namibia
RE16/089	Positive	28.68	Namibia
RE16/116	Positive	28.77	Namibia
RE16/119	Positive	28.80	Namibia
M3	Positive	28.94	MAHC
RE16/077	Positive	28.95	Namibia
RE16/093	Positive	29.01	Namibia
RE16/104	Positive	29.16	Namibia
RE16/101	Positive	29.28	Namibia
RE16/123	Positive	29.51	Namibia
RE16/086	Positive	29.69	Namibia
RE16/071	Positive	29.71	Namibia
RE16/085	Positive	30.44	Namibia
RE16/107	Positive	31.00	Namibia
RE16/080	Positive	31.00	Namibia
RE16/097	Positive	31.73	Namibia
RE16/100	Positive	31.95	Namibia
RE16/070	Positive	32.81	Namibia
RE16/083	Positive	33.72	Namibia
RLB16_003	Positive	25.16	OVAH
M4	Positive	29.34	MAHC
RLB19_053	Positive	29.46	OVAH
M2	Positive	30.74	MAHC
RLB19_080	Positive	31.35	OVAH
OVAH 2	Positive	33.27	OVAH
OVAH 3	Positive	33.72	OVAH
RLB1123_14	Positive	34.92	OVAH
RE16/075	Negative	28.34	Namibia
RE16/087	Negative	32.98	Namibia
RE16/103	Negative	35.93	Namibia

RE16/115	Negative	Negative	Namibia
RE16/122	Negative	Negative	Namibia
RE16/082	Negative	Negative	Namibia
RE16/090	Negative	Negative	Namibia
RE16/092	Negative	Negative	Namibia
RE16/094	Negative	Negative	Namibia
RE16/096	Negative	Negative	Namibia
RE16/102	Negative	Negative	Namibia
M8	Negative	Negative	MAHC
RE16/069	Negative	Negative	Namibia
RE16/072	Negative	Negative	Namibia
RE16/074	Negative	Negative	Namibia
RE16/078	Negative	Negative	Namibia
RE16/111	Negative	Negative	Namibia
M1	Negative	Negative	MAHC
M5	Negative	Negative	MAHC
M6	Negative	Negative	MAHC
M7	Negative	Negative	MAHC
M9	Negative	Negative	MAHC
OVAH 1	Negative	Negative	OVAH
OVAH 4	Negative	Negative	OVAH
RE16/099	Negative	Negative	Namibia
RE16/105	Negative	Negative	Namibia
RE16/106	Negative	Negative	Namibia
RE16/109	Negative	Negative	Namibia
RE16/114	Negative	Negative	Namibia
RE16/118	Negative	Negative	Namibia
RE16/120	Negative	Negative	Namibia
RE16/121	Negative	Negative	Namibia
RLB19_001	Negative	Negative	OVAH
RLB19_009	Negative	Negative	OVAH
RLB19_010	Negative	Negative	OVAH
RLB19_011	Negative	Negative	OVAH
RLB19_012	Negative	Negative	OVAH
RLB19_052	Negative	Negative	OVAH
RLB19_060	Negative	Negative	OVAH
RLB19_020	Negative	Negative	OVAH
RLB19_014	Negative	Negative	OVAH
RLB19_015	Negative	Negative	OVAH
RLB19_016	Negative	Negative	OVAH
RLB19_017	Negative	Negative	OVAH
RLB19_018	Negative	Negative	OVAH
RLB19_004	Negative	Negative	OVAH
RLB18_214	Negative	Negative	OVAH
RLB18_105	Negative	Negative	OVAH
RLB18_092	Negative	Negative	OVAH
RLB17_002	Negative	Negative	OVAH
RLB17_117	Negative	Negative	OVAH
RLB18_081	Negative	Negative	OVAH
RLB18_086	Negative	Negative	OVAH
RLB18_074	Negative	Negative	OVAH

RLB18_072	Negative	Negative	OVAH
RLB18_071	Negative	Negative	OVAH
RLB18_070	Negative	Negative	OVAH
RLB18_068	Negative	Negative	OVAH
RLB18_067	Negative	Negative	OVAH
RLB18_064	Negative	Negative	OVAH
RLB18_066	Negative	Negative	OVAH
RLB18_063	Negative	Negative	OVAH
RLB18_062	Negative	Negative	OVAH
RLB18_060	Negative	Negative	OVAH
RLB18_059	Negative	Negative	OVAH
RLB18_053	Negative	Negative	OVAH
RLB18_058	Negative	Negative	OVAH
RLB18_049	Negative	Negative	OVAH
RLB18_048	Negative	Negative	OVAH
RLB16_165	Negative	Negative	OVAH
RLB19_061	Negative	Negative	OVAH
RLB19_066	Negative	Negative	OVAH
RLB19_068	Negative	Negative	OVAH
RLB19_069	Negative	Negative	OVAH
RLB19_070	Negative	Negative	OVAH
RLB19_071	Negative	Negative	OVAH
RLB19_072	Negative	Negative	OVAH
RLB19_074	Negative	Negative	OVAH
RLB19_075	Negative	Negative	OVAH
RLB19_076	Negative	Negative	OVAH
OVAH 5	Negative	Negative	OVAH
RE16/095	Negative	Negative	Namibia

8.2 Owner's consent form

Consent form

Name of owner:
Case No:
Date:

INFORMED CONSENT FORM

We, the undersigned, hereby agree that the animal(s), as specified below, may be used by the researcher(s), as specified below, in the procedures as explained below:

Completed by the researcher(s)

NAME OF THE RESEARCHER(S):

Nokuzola Faith Nkosi (student)
Melvyn Quan (Supervisor)

NAME OF RESEARCH PROJECT:

Development and validation of group and species-specific real-time PCR and xMAP® assays to detect *Ehrlichia/Anaplasma* spp. affecting dogs.

PURPOSE OF RESEARCH PROJECT:

Optimise existing group and species-specific real-time PCR assays to detect *Ehrlichia/Anaplasma* spp. in dogs. Develop and optimise new group and species-specific real-time PCR assays to detect *Ehrlichia/Anaplasma* spp. in dogs. Convert the real-time assays to xMAP assays and validate.

DETAILED PROCEDURE(S) TO BE PERFORMED:

Dog will be physical restrained for taking blood sample by sticking a needle into the jugular vein and collecting the blood into tubes containing anticoagulant, according to Onderstepoort Veterinary Academic Hospital (OVAH) standard operating procedure. This should only cause minimal distress, as the procedure will be of short duration and not painful. Procedure is expected to be of short duration (< 1 min).

RISK(S) INVOLVED IN SPECIFIED PROCEDURE: None

IDENTIFICATION OF ANIMAL TO BE USED: Clinic records

To be completed by the animal's owner or person duly authorized to sign on his/her behalf:

The undersigned parties further agree that no compensation will be payable to the animal's owner or anybody else and that all research associated costs will be covered by the researcher(s).

The undersigned parties further agree that this form would serve to fully indemnify the University of Pretoria and the undersigned researcher(s) against any future claims resulting from the specified procedure by or on behalf of the animal's owner.

The undersigned parties further agree that no material of any kind, including data and research findings, obtained or resulting from the procedure, would be passed on to any third party or used for any purpose other than that specified in this form, except with the written consent of the undersigned owner of the animal.

Unmistakeable distinguishing description of animal to be used:

Have you received detailed information regarding the proposed study?	**Yes/ No**
Have all the risks involved in the procedure been explained to you and do you fully understand these risks?	**Yes/ No**
Do you grant full consent for the procedure to be performed?	**Yes/ No**

_____ _____

Signature of owner Signature of clinician/Student

8.3 Donor dog full blood count

08/27/2018 11:49 AM
P4387252

VETERINARY DIAGNOSTIC LABORATORY
P/BAG X04, ONDERSTEPOORT, 0110
Request/Result Detail - CLR0001

Client
QUAN, MELVYN
DEPARTMENT OF VETERINARY TROPICAL DISEASES
FACULTY OF VETERINARY SCIENCES
PRETORIA GP 0110
W 125298142

Patient
CANINE 1
GREYHOUND
CANINE

Completed 21 of 21 Results

Accession #	Item	Priority	Status		Dates
D-CP09661-18	CBC	ROUTINE	COMPLETE	**Requested:**	08/27/2018 11:31am
Requesting DVM:	UNKNOWN			**Collection:**	08/27/2018 11:31am
Student:				**Received:**	08/27/2018 11:38am
Comments:				**Needed:**	08/27/2018 11:31am
				Status:	08/27/2018 11:47am

. - HAEMATOLOGY

TEST	RESULT		UNITS	REF RANGE	RESULT DATETIME
HAEMOGLOBIN	230	H	g/L	120 - 180	08/27/2018 11:43am
RED CELL COUNT	9.84	H	x10^12/L	5.5 - 8.5	08/27/2018 11:43am
HAEMATOCRIT	0.69	H	L/L	.37 - .55	08/27/2018 11:43am
MEAN CORPUSCULAR VOLUME	69.7		fL	60 - 77	08/27/2018 11:43am
MEAN CORPUSCULAR HAEMOGLOBIN	23.4		pg	No Reference Range	08/27/2018 11:43am
MEAN CORPUSCULAR HAEMOGLOBIN CONCENTRATION	33.5		g/dL	32 - 36	08/27/2018 11:43am
RED CELL DISTRIBUTION WIDTH	12.4		%	No Reference Range	08/27/2018 11:43am
WHITE CELL COUNT	4.61	L	x10^9/L	6 - 15	08/27/2018 11:44am
SEGMENTED NEUTROPHIL	2.21	L	x10^9/L	3 - 11.5	08/27/2018 11:44am
BAND NEUTROPHIL	0.00		x10^9/L	0 - .5	08/27/2018 11:44am
LYMPHOCYTE	0.85	L	x10^9/L	1 - 4.8	08/27/2018 11:44am
MONOCYTE	0.32		x10^9/L	.15 - 1.35	08/27/2018 11:44am
EOSINOPHIL	1.22		x10^9/L	.1 - 1.25	08/27/2018 11:44am
BASOPHIL	0.00		x10^9/L	0 - .1	08/27/2018 11:45am
OTHER	0.00		%	No Reference Range	08/27/2018 11:45am
PLATELET COUNT	143	L	x10^9/L	200 - 500	08/27/2018 11:45am
NRBC/100 WBC	0			0 - 9	08/27/2018 11:45am
MORPHOLOGY	2+ ANISOCYTOSIS, MILD MACROCYTOSIS, FEW REACTIVE LYMPHOCYTES			No Reference Range	08/27/2018 11:46am
PLATELET COMMENT	LOW PLATELET COUNT ON SMEAR, MILD PLATELET AGGREGATION			No Reference Range	08/27/2018 11:46am
PARASITE ID	NO PARASITES SEEN ON SMEAR			No Reference Range	08/27/2018 11:47am
SAMPLE CONDITION	CLEAR			No Reference Range	08/27/2018 11:46am

Comments:

HAEMATOCRIT	This result has been verified
WHITE CELL COUNT	This result has been verified
LYMPHOCYTE	This result has been verified
LYMPHOCYTE	This result has been verified
LYMPHOCYTE	This result has been verified
LYMPHOCYTE	This result has been verified
EOSINOPHIL	The eosinophils have non-staining granules consistent with "ghost eosinophils" which have been well-described in this breed.
PLATELET COUNT	This result has been verified
MORPHOLOGY	FRAGILE WBC3+

8.4 Bayesian latent class model

```
model{
  y1[1:Q, 1:Q] ~ dmulti(p1[1:Q, 1:Q], n1)
  y2[1:Q, 1:Q] ~ dmulti(p2[1:Q, 1:Q], n2)
  p1[1,1] <- pi1*eta11 + (1-pi1)*theta11
  p1[1,2] <- pi1*eta12 + (1-pi1)*theta12
  p1[2,1] <- pi1*eta21 + (1-pi1)*theta21
  p1[2,2] <- pi1*eta22 + (1-pi1)*theta22
  p2[1,1] <- pi2*eta11 + (1-pi2)*theta11
  p2[1,2] <- pi2*eta12 + (1-pi2)*theta12
  p2[2,1] <- pi2*eta21 + (1-pi2)*theta21
  p2[2,2] <- pi2*eta22 + (1-pi2)*theta22
  eta11 <- lambdaD*eta1
  eta12 <- eta1 - eta11
  eta21 <- gammaD*(1-eta1)
  eta22 <- 1 - eta11 - eta12 - eta21
  theta11 <- 1 - theta12 - theta21 - theta22
  theta12 <- gammaDc*(1-theta1)
  theta21 <- theta1 - theta22
  theta22 <- lambdaDc* theta1
  eta2 <- eta11 + eta21
  theta2 <- theta22 + theta12
  rhoD <- (eta11 - eta1*eta2) / sqrt(eta1*(1-eta1)*eta2*(1-eta2))
  rhoDc <- (theta22 - theta1*theta2) / sqrt(theta1*(1-theta1)*theta2*(1-theta2))
  pi1 ~ dbeta(1.46, 6.24) ##mode=0.08, 5th%ile=0.01
  pi2 ~ dbeta(5.03, 7.04) ##mode=0.40, 5%ile=0.2
  eta1 ~ dbeta(23.9, 5.04) ##mode=0.85, 5%ile=0.7
  theta1 ~ dbeta(22.98, 3.44) ##mode=0.9, 5%ile=0.75
  lambdaD ~ dbeta(1.94, 1.10) ## Mode=0.90, 5th%tile=0.20
  gammaD ~ dbeta(1.94, 1.10)
  lambdaDc ~ dbeta(1.94, 1.10) ## Mode=0.90, 5th%tile=0.20
  gammaDc ~ dbeta(1.94, 1.10)
}

list(n1=66, n2=55, Q=2,
y1=structure(.Data=c(
9,0,0,57),.Dim=c(2,2)),
y2=structure(.Data=c(
31,0,2,22),.Dim=c(2,2)))
list(pi1=0.08, pi2=0.4, eta1=0.85, theta1=0.90, lambdaD=0.50, lambdaDc=0.50,
gammaD=0.50, gammaDc=0.50)
```

8.5 Standard operating procedure for *E. canis*

TITLE :	***E. canis* real-time PCR testing**
REFERENCE:	

1. PURPOSE

1.1. To perform real-time PCR for *E. canis* on total nucleic acid extracts from Whatman® FTA cards and blood samples.

2. SCOPE

2.1. To test samples for the detection of nucleic acid for *E. canis.*

3. RESPONSIBILITY AND AUTHORITY

3.1. It is the responsibility of a competent designated technologist to perform the procedures described in this SOP.

3.2. The First Technologist has the responsibility and authority to ensure that this procedure is being adhered to.

3.3. The Laboratory Head has the overall responsibility for technical operations in the laboratory.

4. EQUIPMENT

4.1. **Area 1: Master Mixture – Room 2-64.1**

4.1.1. Freezer at -20°C (± 2°C).

4.1.2. Biohazard Cabinet.

4.1.3. Single channel Micropipettes (2.5 µl / 10 µl / 100 µl / 200 µl / 1000 µl) (See SOP QA/Mol/SOP 1.5.1.8.1 – 1.5.1.10.5- How to use a single channel micropipette).

4.1.4. Mini Centrifuge (large and small tube with adaptors).

4.1.5. Vortex (Asset #: 753643).

4.2. **Area 2: Extraction Lab – Room 2-81**

4.2.1. Fridge at 4°C (±2°C).

4.2.2. Freezer at -20°C (± 2°C).

4.2.3. Biohazard safety cabinet.

4.2.4. Laminar flow cabinet.

4.2.5. Single channel micropipettes (2.5 µl / 10 µl / 100 µl / 200 µl / 1000 µl).

4.2.6. Mini centrifuge (large and small tube with adaptors) (asset #: 910867)

4.2.7. Vortex (Asset #: 0474140).

4.2.8. ThermoScientific Kingfisher Duo Prime Machine (Asset #: 928806).

4.3. **Area 3: PCR Machine Laboratory – Room 2-81.2**

4.3.1. ABI StepOnePlus RT-PCR System machine (Asset #: 849187).

5. MATERIALS

5.1. Permanent marker pen.

5.2. Green eppendorf / test tube racks (Room 2-64.1).

5.3. Blue eppendorf / test tube racks (room 2-81).

5.4. Sterile filter tips (10 µl / 100 µl / 200 µl / 1000 µl - supplied by Lasec).

5.5. Plastic beaker (Glassware store – room 2-72.1.1).

5.6. 0.1 MicroAmp Fast Reaction Tubes (8 tubes / Strips) (Supplied by ThermoFisher; Cat #: 4358293) **OR** ABI 0.1 Fast Optical PCR Plate (Supplied by: ThermoFisher/ Cat #: 4346906).

5.7. KingFisher Deep Well 96 well Extraction Plate (ThermoFisher / Cat #: 95040460).

5.8. KingFisher DUO 12-Tip Comb (ThermoFisher / Cat #: 97003500).

5.9. Eppendorf tubes (2 ml / 1.5 ml) / sterile specimen containers.

5.10. Sterile McCartney bottles.

5.11. Gloves.

5.12. Sterile scissors.

5.13. Crushed ice.

6. REAGENTS

6.1. NB: Reagents should be re-constituted according to manufacturer's instructions. Any deviations should be validated.

6.2. 2x qPCR Master Mix.

6.3. 12.5x *E. canis* primer/probe mix.

6.3.1. Forward primer– AnapEhrlichia_F (20 µM) - (5'-AGC YTA ACA CAT GCA AGT CGA A-3').

6.3.2. Reverse primer – AnapEhrlichia_R (20 µM) - (5'-TTA GTG GCA GAC GGG TGA GTA A-3').

6.3.3. Probe – *E. canis*_P (20 µM) - (*FAM*- AGC CTC TGG CTA TAG GA-*MGB).*

6.4. Nuclease-free H_2O.

6.5. Applied Biosystems MagMax CORE Nucleic Acid Purification Kit

6.5.1. Wash solution 1

6.5.2. Wash solution 2

6.5.3. Lysis Buffer

6.5.4. Binding Solution

6.5.5. Nucleic Acid Binding Beads

6.5.6. Proteinase K
6.5.7. Elution Buffer
6.6. PBS – pH 7,4

7. DOCUMENTATION

7.1. SOP: QA/Mol/SOP 1.5.1.81 – 1.5.1.10.5 – How to use a single and multi- channel micropipette.
7.2. SOP: QA/Mol/SOP 1.5.2.20 – Operating instructions for the Biohazard Safety cabinet 4 Class II (CRT).
7.3. SOP: QA/Mol/SOP 1.5.1.2 & 1.5.1.3 – Operating instructions for Biohazard Safety Cabinet Class II Linear Scientific 1 & Laminaire 2.
7.4. SOP: QA/Mol/SOP 1.5.1.11 – Operating instructions for the Wealtec E-bench Centrifuge.
7.5. SOP: QA/Mol/SOP 1.5.1.6 – Operating instructions for vortex mixer 1 Biocote Stuart® SA8.
7.6. FORM: QA/FORM/MOL 4.1 – *E. canis* Mastermix / Sample preparation Form.
7.7. Logbook: QA/MOL/LOG 001 – Area 1 Equipment logbook Room 2-64.1.
7.8. Logbook: QA/MOL/LOG 002 – Area 2 Equipment logbook Room 2-81.
7.9. Logbook: QA/MOL/LOG 003 – Area 3 Equipment logbook Room 2-81.1.

8. SAFETY

8.1. **Safety measures**
8.1.1. Always wear gloves.
8.1.2. NEVER move from area 3 back to area 2 or area 1. Never move from area 2 back to area 1.
8.1.3. Safety data sheets located in safety data sheet file in room 2-54.
8.2. **Precautionary measures**
8.2.1. All reagents must be stored at -20°C ± 2°C at all times when not in use.
8.2.2. All reagents to be mixed well before use.
8.2.3. Remove only a sufficient amount of reagents from the freezer for use.
8.2.4. New tips must be used for each sample.

9. PROCEDURE

9.1. **PCR Master Mix set-up (Area 1) – Room 2-64.1:**
9.1.1. Determine the amount of samples to be tested and add 2-3 reactions. (e.g. 10 samples to be tested, add 3 reactions to make sure you prepare enough PCR Mix, thus make your mix up for 13 reactions).
9.1.2. Take reagents from freezer to thaw.
9.1.3. Wipe biohazard cabinet with Corox disinfectant.

9.1.4. Make up Master Mix (according to QA/FORM/MOL 3.1 and 4.1) in 1.5ml eppendorf tubes.

Primer/Probe	Sequence 5' - 3'
AnapEhrlichia_F121_142	AGC YTA ACA CAT GCA AGT CGA A
AnapEhrlichia_R180_201	TTA GTG GCA GAC GGG TGA GTA A
Ecanis_P158_174	AGC CTC TGG CTA TAG GA

	×1 (µl)	×…
TaqMan Fast Advanced Master Mix	10.00	
Forward primer - AnapEhrlichia_F (20 µM)*	0.20	
Reverse primer - AnapEhrlichia_R (20 µM)*	0.20	
Probe – E canis FAM (20 µM)*	0.25	
Nuclease-free H2O	7.35	
DNA template	2.00	
Total volume	20.00	

9.1.5. Aliquot the required amounts of master mix (18 µl) to the PCR strip tubes or 96 well plate.

9.1.6. Close with strip flat caps or seal plate with foil seal.

9.1.7. Place tubes or plates on ice and move to area 2 for DNA extraction.

9.2. **Extraction (Area 2) – Room 2-81:**

9.2.1. Prepare the Lysis Binding solution per sample: Lysis buffer (350 µl) and Binding solution (350 µl).

9.2.2. Prepare the Bead Mixture per sample: Beads (20 µl) and Proteinase K (10 µl).

9.2.3. In a 96 deep well plate add as follows:

9.2.3.1. Row B: 500 µl washing buffer 1.

9.2.3.2. Row C: 500 µl washing buffer 2.

9.2.3.3. Row H: Add tip comb.

9.2.3.4. In separate row: Add an elution strip.

9.2.3.5. Add 90 µl Elution buffer to the elution strip.

9.2.3.6. Add 30 µl of the bead mix in Row A of the 96 deep well plate.

9.2.3.7. Add 200 µl of the sample to row A and mix with the same pipette tip.

9.2.3.8. Add 700 µl of Lysis/binding solution mix also to Row A and mix with the same tip.

9.2.4. Load the plate onto the KingFisher Duo Prime machine (Row A right top corner) and place the Elution Strip at the bottom and close the lever.

9.2.5. Select the testing programme: DNA/RNA → CORE duo → Enter (√) → Start (►) → Press Enter (√) to continue (± 30 minutes / plate).

9.2.6. When extraction is finished, load the DNA into your Master Mix strips.

9.2.7. Seal with a new cap-strip.

9.3. **PCR Machine Lab (Area 3) – Room 2.81.1:**

9.3.1. Switch computer and StepOnePlus machine on.

9.3.2. Password = User name

9.3.3. Double click on StepOnePlus software icon 2.3

9.3.4. Log-in as guest.

9.3.5. Sample Set-up:

9.3.6. A “set-up”, “run”, “Analyse screen will appear.

9.3.7. Select New Experiment → Advanced Set-up.

9.3.8. Type in an Experiment name

9.3.9. Select StepOnePlus

9.3.10. Select “Experiment Type” by clicking on Quantitation “Standard Curve”

9.3.11. Select reagents used: “TaqMan”

9.3.12. Select ramp speed – “Standard (45 min)”

9.3.13. Click on Plate Set-up (left hand side of screen)

9.3.14. Click on “add saved target” and select the desired targets from the list and click “add targets”

9.3.15. Add samples names by clicking on “define sample” and then on “add new sample”. Add new samples until all samples have been entered.

9.3.16. Click on “assign targets and samples”

9.3.17. Select where the desired samples should be placed according to where they are placed on the PCR machine.

9.3.18. Assign probes to the desired wells in which that specific target should be detected.

9.3.19. Deselect “Use default”

9.3.20. Select “Automatic Threshold” → Threshold 0,1 → Apply

9.3.21. Click on “Run Method” → Open Run method

9.3.21.1. Select Taqman fast advance master mix and click “Yes”

9.3.22. Click on “Save”

9.3.23. Select Data drive (D:), select “Applied Biosystems, select Software 2.3, select experiments 2.3.

9.3.24. Place strips into the StepOnePlus RT-PCR machine using the black strip holder plate. When working with plates, remove the black strip holder.

9.3.25. Start Run

10. Results:

10.1. Select StepOnePlus 2.3

10.2. User name and password = Guest

10.3. Click on File, open and select Experiment 2.3

10.4. Click on saved file name.

10.5. Click on “graph Type” and change the view from “log” to “linear”.
10.6. Click on the drop down box for “Target” below the graph.
10.7. Cut off – 37, thus > 37 = Positive and, 37 = Negative
10.8. Click on “Export”, browse and select file. Click Computer → Kingston → Open → Start Export

11. Quality Control

11.1. Unsuitable samples (e.g. samples that have been exposed to high temperatures) should not be tested.
11.2. Positive and negative controls must be run with each plate of samples tested.
11.3. The test is valid when the positive and negative controls produce the expected results.

12. Waste Disposal

12.1. Samples are discarded into bio-hazard waste bags for incineration or for the removal by an approved waste removal provider and consumables are placed in Corox disinfectant.

13. Forms

13.1. All raw data working sheets pertaining to the test are to be clearly legible in permanent ink and filed and retained for a minimum of 5 years.

8.6 Ethical clearance

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Development and validation of Group and Species - specific real time PCR and xMAP® assays to detect *Ehrlicia/Anaplasma spp.* affecting dogs**
PROJECT NUMBER	**V099-17**
RESEARCHER/PRINCIPAL INVESTIGATOR	**FN Nkosi**

STUDENT NUMBER (where applicable)	**U04906480**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPESIES	**Dogs**	
NUMBER OF ANIMALS	**25**	
Approval period to use animals for research/testing purposes		**September 2017 – September 2018**
SUPERVISOR	**Prof. M Quan**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 26 September 2017
CHAIRMAN: UP Animal Ethics Committee	Signature

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

Extension No. 1

PROJECT TITLE	**Development and validation of Group and Species - specific real time PCR and xMAP® assays to detect *Ehrlicia/Anaplasma spp.* affecting dogs**
PROJECT NUMBER	**V099-17**
RESEARCHER/PRINCIPAL INVESTIGATOR	**FN Nkosi**

STUDENT NUMBER (where applicable)	**U_04906480**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPESIES	**Dogs**	
NUMBER OF ANIMALS	**9 used**	
Approval period to use animals for research/testing purposes		**March 2018 – March 2019**
SUPERVISOR	**Prof. M Quan**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 4 April 2018
CHAIRMAN: UP Animal Ethics Committee	Signature

8.7 DAFF section 20 approval

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Henry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HenryG@daff.gov.za
Reference: 12/11/1/1/6

Ms Faith Nokuzola Nkosi
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria

Dear Ms Nkosi,,

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Your fax / memo / letter/ Email dated 25 0ctober 2017, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. The study may only start after having obtained ethics approval;
4. Samples may only be collected from suspected clinical cases of *Ehrlichia/Anaplsama* that are presented to the Onderstepoort Veterinary Academic Hospital;
5. No field samples may be collected;
6. The Waste Group must be used as accredited waste management company;
7. No materials from this study may be disposed of at a land fill site;
8. This approval is only valid until 31 December 2018.

Title of research/study: Development and Validation of Group and Species-specific Real Time PCR and xMAP Assays to Differentiate *Ehrlichia/Anaplasma* spp. Affecting Dogs
Researcher (s): Ms Faith Nokuzola Nkosi
Institution: Faculty of Veterinary Science, UP

Your Ref./ Project Number:
Our ref Number: 12/11/1/1/6

Kind regards,

DR. MPHO MAJA

DIRECTOR OF ANIMAL HEALTH

Date: 2017 -11- 17