Detection and partial molecular characterization of *Rickettsia* and *Bartonella* from southern African bat species

by

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I declare that the thesis, which I hereby submit for the degree MSc (Microbiology) at the University of Pretoria, South Africa, is my own work and has not been submitted by me for a degree at another university

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Tjale Mabotse Augustine

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Summary

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In Africa, bats have been implicated in a number of emerging and re-emerging diseases, mostly associated with viral infections – such as caused by rabies-related lyssaviruses, paramyxoviruses and coronaviruses. Whereas most infectious agents reported in bats have been viruses, bacterial species have rarely been reported. The main objective of this study was to identify bacterial species that may circulate in southern African bats by using nucleic acid detection methods. Two bacterial species were targeted: *Rickettsia* and *Bartonella.* These were chosen because they are capable of infecting and causing diseases in a wide range of hosts including humans.

We evaluated and optimized several polymerase chain reaction (PCR) assays to detect *Rickettsia* and *Bartonella*. These included PCRs targeting the citrate synthase gene (*gltA)* and *16S rRNA* gene for *Rickettsia* and the citrate synthase (*gltA*) gene for *Bartonella.* A panel of 354 bat blood samples, collected from different sites in South Africa and Swaziland, were tested using these assays. *Rickettsia* and *Bartonella* DNA was detected in 6/354 and 13/354 bats, respectively, and characterized using DNA sequencing. All the *Rickettsias* were closely related to

other *Rickettsia* species circulating in these areas and all the *Bartonellas* clustered together, but were distantly related to *Bartonella* species from the same geographical area.

This study reports for the first time the detection of *Rickettsia* and *Bartonella* DNA in southern African bats. This finding contributes to the knowledge regarding *Rickettsia* and *Bartonella* diversity and host distribution. The epidemiology and transmission pathways of these bacteria in bat populations remains to be elucidated as is the public health importance of the circulation of these potential pathogens in bats. A likely source of infection is unknown, but since bats carry ectoparasites (flies, fleas, ticks and mites); surveillance for these pathogens in ectoparasites should be a first step in elucidating epidemiology and transmission pathways to other hosts including humans. Given the potential for human disease, the surveillance and characterization of these pathogens will be in the interest of good public health practices.

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List of abbreviations

ATBF: African tick bite fever

ATP: Adenosine triphosphate

AG: Ancestral group

bp: Base pair

BHQ: Black hole quencher

BLAST: Basic local alignment search tool

CD4: Cluster of differentiation 4

CD8: Cluster of differentiation 8

CSD: Cat scratch disease

CNI: the close-neighbour interchange

CV: Coefficient variation

DNA: Deoxyribonucleic acid

ELISA: Enzyme-linked immunosorbant assay

FISH: Fluorescent *in situ* hybridation

FtsZ: Cell replication gene

gltA: Citrate synthase gene

GroEL: Heat shock encoding gene

IDT: Intergrated DNA Technology

IFA: Indirect immune fluorescent assay

IFN-γ: Interferon gamma

IgG: Immunoglobin G antibody

IgM: Immunoglobin M antibody

IL-β: Interleukin-1 beta

LPS: Lipopolyssacharide

MSF: Mediterranean spotted fever

MST: Multi spacer typing

MP: Maximum parsimony

NADH: Nicotinamide adenine dinucleotide plus hydrogen

NCBI: National Centre for Biotechnology Information

OmpA: Outer membrane protein encoding gene A

OmpB: Outer membrane protein encoding gene B

PE: Peliosis hepatis

RibC: Riboflavin synthase gene

RMSF: Rocky Mountain spotted fever

rRNA: Ribosomal ribionucleic acid

SD: Standard deviation

SFG: Spotted fever group

SOC: Super optimal culture

spp: Species

STG: Scrub typhus

SPA: Surface protein antigens

TG: Typhus group

TMR: Tetramethylrhodamine

TNF-α: Tumor necrosis factor- alpha

Chapter 1: Literature review

1.1 General introduction

Over the past decade, there has been an increasing interest concerning the role of bats as reservoirs of zoonotic diseases (Calisher *et al.,* 2005; Wong *et al.,* 2007). Bats have been implicated with various zoonotic viruses including rabies and rabies-related lyssaviruses (Markotter *et al*., 2006; Kuzmin *et al.,* 2008), paramyxoviruses (Halpin *et al*., 2000), SARS-related coronaviruses (Li *et al*., 2005) and Marburg viruses (Towner *et al.,* 2009; Kuzmin *et al*., 2010b). Because bats are highly mobile, have unique social behaviours (communal roosting), have relatively long life span for their size, are broadly distributed geographically and prone to ectoparasites infestation, they are highly favorable to host and vector infectious diseases (Wong *et al.,* 2007).

Increased tourism attraction to caves, alterations of natural habitats (forests and caves) of bats and migration of bats to urban areas as a result of urban expansion in Africa, has resulted in a closer association of certian bat species with humans (Wong *et al.,* 2007). In Africa, zoonotic diseases associated with bats have been reported (Raoult *et al*., 2001; Cleaveland *et al*., 2001). These include Duvenhage virus in southern Africa (Markotter *et al.,* 2006), henipah viruses in Ghana (Drexler *et al.,* 2009) and Marburg virus and SARS-like coronaviruses in Kenya (Tong *et al*., 2009; Kuzmin *et al.,* 2010b). These studies do however present isolated findings and the extent and importance of bat-harboured zoonoses on public and veterinary health remains to be fully appreciated. The focus of pathogen discovery studies in recent years have been viral, and very few studies have investigated the bacterial infections, especially of potential zoonotic nature of bats (Ghatak *et al*., 2000; Reeves *et al*., 2006a, Evans *et al.,* 2009; Childs-Sandfords *et al.,* 2009).

Members within the genus *Bartonella* and *Rickettsia* have common features; both fall under the sub class α-proteobacteria, Gram negative, transmitted to a susceptible host by blood sucking arthropods and are intracellular organisms that require a host for multiplication. *Bartonella* and *Rickettsia* species have been reported in a wide variety of hosts including rodents, ruminants, felines and canines but have rarely been detected in bats (Perine *et al.,* 1992; Kelly *et al.,* 1998; Pretorius *et al.,* 2004(a); Shaw *et al.,* 2004; Molia *et al.,* 2004; Nicholson *et al.,* 2010; Kelly *et al.,* 2010). Although few studies have reported the detection of nucleic acid or antibody to

Bartonella in bats (Kosoy *et al*., 2010; Bai *et al.,* 2011, Lin et al., 2011), there is no study to date that has reported detection of rickettsial DNA in bats. These pathogens are associated with an increasing continuum of emerging and re-emerging diseases (Sackal *et al*., 2008; Frean *et al*., 2008; Kerniff *et al*., 2010; Mediannikov *et al*., 2010; Socolovschi *et al.,* 2010). Two studies reported sero-positivity against rickettsial antigens in specific bat populations (Reeves *et al.,* 2006a; DÁuria *et al*., 2010), but prevalence and incidence of *Rickettsia* in the broader bat populations remain unclear. On the other hand, *Bartonella* has been detected using specific PCR assay and/or isolated in numerous bats populations from Taiwan, Kenya, Guatemala and Europe, respectively (Concannon *et al*., 2005; Kosoy *et al*., 2010, Bai *et al.,* 2011; Lin *et al.,* 2011).

The aim of this study was firstly to evaluate and compare PCR assays for the detection of *Rickettsia a*nd *Bartonella.* Such assays will be valuable as diagnostic and surveillance tools. Secondly, these assays were used to detect *Bartonella* and *Rickettsia* genomic DNA in a panel of bat blood specimens. This panel represented different bat species collected from South Africa and Swaziland.

1.2 *Rickettsia*

Members of the genus *Rickettsia* are obligate intracellular bacteria that are transmitted to humans and other vertebrates by blood-sucking arthropods such as lice, fleas, mites and ticks (Brenner *et al*., 2005). The genus *Rickettsia* was previously divided antigenically into three groups: Spotted fever group (SFG), typhus group (TG) and scrub typhus group (STG) (Parola and Rauolt*,* 2007; Fournier and Raoult, 2009). However, scrub typhus *Rickettsia* belongs to the separate genus *Orientia* on the basis of *16S rRNA* gene sequence analysis and by lacking lipopolysaccharide, peptidoglycan and a slime layer (Walker, 2007; Fournier and Raoult, 2009). Currently the genus *Rickettsia* comprises of 25 validated species and 14 of these cause human infections (Fournier and Raoult, 2009; Merhej and Rauolt, 2011).

1.2.1 *Rickettsia* taxonomy and phylogeny

The term *Rickettsia* has been previously linked with small and uncultivable bacteria, but due to the introduction of new classification techniques, this term applies to the arthropod-borne bacteria, which belong under the genus *Rickettsia,* within the family

Rickettsiaceae, in the order *Rickettsiales* and the sub-order α-proteobacteria (Parola and Rauolt, 2007).

Currently the order *Rickettsiales* comprises of the genera *Anaplasma*, *Ehrlichia*, *Neorickettsia*, *Orientia*, *Rickettsia* and *Wolbachia* (Fournier and Raoult, 2009). Figure 1.1 indicates the bacterial species under the order *Rickettsiales.* The genus *Rickettsia* comprises of 25 different species and it is divided phylogenetically into four groups of bacteria: Spotted fever group (*R. conorii*, *R. africae*, *R. ricketttsii* and other spotted fevers), typhus group (*R. typhii* and *R. prowazekii*), transitional group (*R. akari* and *R. felis*) and ancestral group (*R. bellii*) (Gillespie *et al*., 2007; Fournier and Rauolt, 2009).

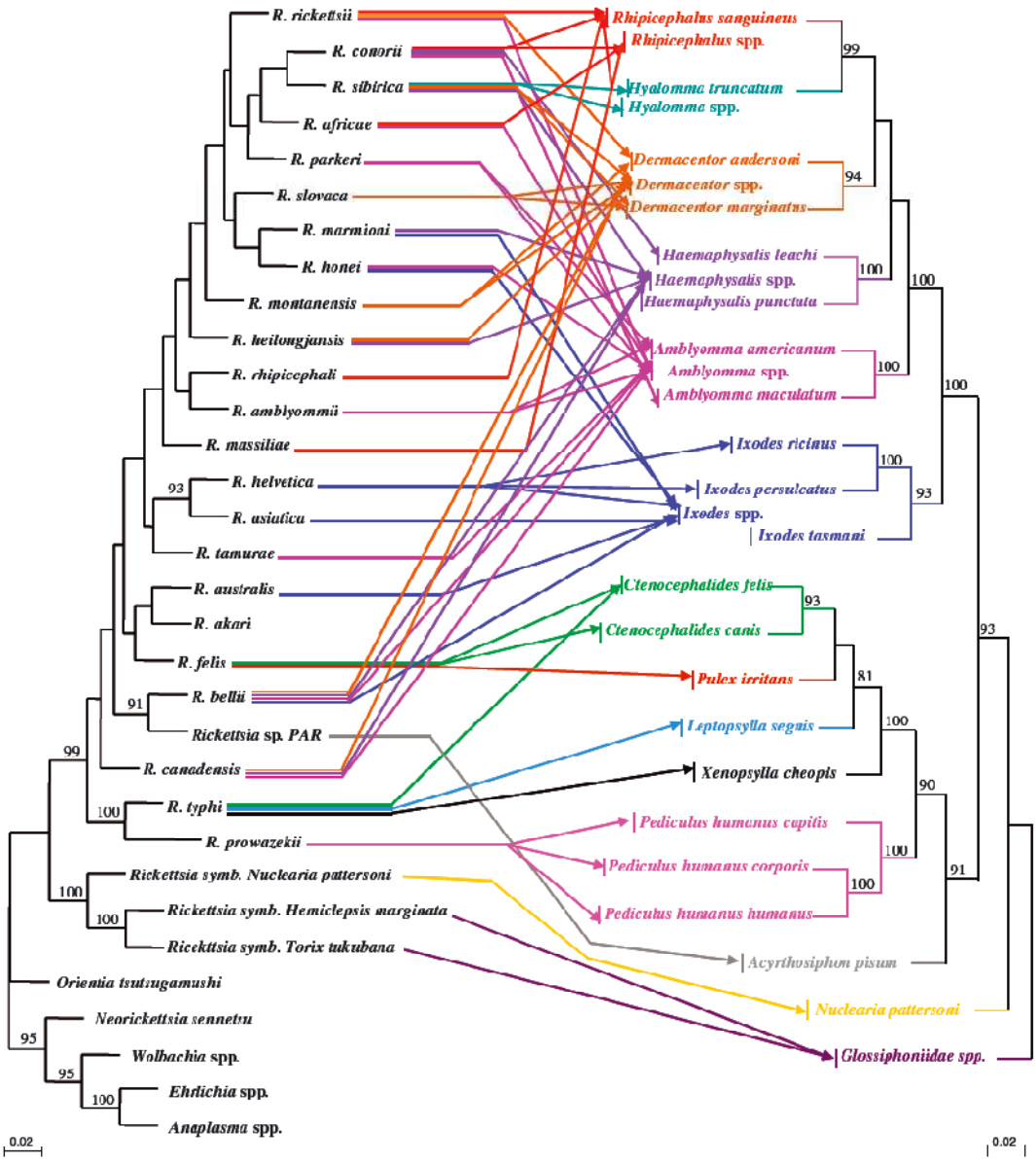


Figure 1.1: Phylogenetic relationships of the organisms of the order *Rickettsiales* and their reservoir hosts based on *16S rRNA* and *18S rRNA* gene sequences. Only the bootstrap values higher than 75% are shown in the branch nodes. From Merhej and Raoult (2011), license number: 2878240534804.

Originally, classification of *Rickettsia* was based on morphological, antigenic and metabolic characteristics. These characteristics were unreliable for phylogenetic studies of *Rickettsia* (Fournier and Raoult, 2009). Several techniques were suggested; DNA-DNA hybridization (Wayne *et al.,* 1987), average nucleotide identity (Konstantidinitis and Tiedje*,* 2005), mouse serotyping, SDS-PAGE and pulsed field gel electrophoresis (PFGE) (Roux and Rauolt, 1995). These techniques were not useful, because they either could not discriminate *Rickettsia* species or were not reproducible. The introduction of molecular techniques (PCR and nucleotide sequencing) allowed more appropriate estimation of phylogenetic relationships of *Rickettsia;* with the conserved *16S rRNA* as the gene of choice for these studies (Weinsburg *et al*., 1989; Brenner *et al.,* 1993; Tamura *et al*., 1995; Roux and Raoult, 1995; Roux *et al.,* 1997). Phylogenetic studies based on the *16S rRNA* genes have shown that several bacteria such as *Coxiella burnetti, Rickettsiella grylli* and *Eperytrozoon spp*. do not belong to the α-subgroup of the phylum proteobacteria (Merhej and Raoult, 2011).

Subsequent phylogenetic studies led to the discovery of several genes that can be used for detection and differentiation of *Rickettsia* species. Those genes include the citrate synthase *(gltA*) (Roux *et al.,* 1997), the 17 kDA common antigen (Anderson and Tzianabos, 1989), surface exposed high molecular weight antigenic proteins of the Sca family: *ompA* (Fournier *et al.,* 1998), *ompB* (Roux and Raoult*,* 2000), *sca4* (Sekeyova *et al.,* 2001), *sca1* (Ngwamidiba *et al.,* 2006) and s*ca2* (Ngwamidiba *et al.,* 2005). However, identification of bacterial species should not only rely on one gene and a combination or minimum of five genes should be used (multi locus sequence typing, MSLT) (Stackebrandt *et al*., 2002). MSLT has been developed for classification of *Rickettsia* at the genus, group and species level (Fournier and Raoult*,* 2009). Figure 1.2 summarizes the most recent taxonomic system for classification of *Rickettsia* at the genus and group level (Fournier *et al.,* 2003; Fournier and Rauolt, 2009). This highlights the use of genotypic, phenotypic and phylogenetic characteristics to classify *Rickettsia* to the species level. Currently, there is no gene that can identify *Rickettsia* to the strain level (Fournier and Raoult, 2009). However, the use of multi-spacer typing was effective. This technique relies on the hypothesis that non-coding sequences go through less evolutionary pressure when compared to the coding sequences and are more variable between strains of bacteria (Fournier *et al.,* 2007).

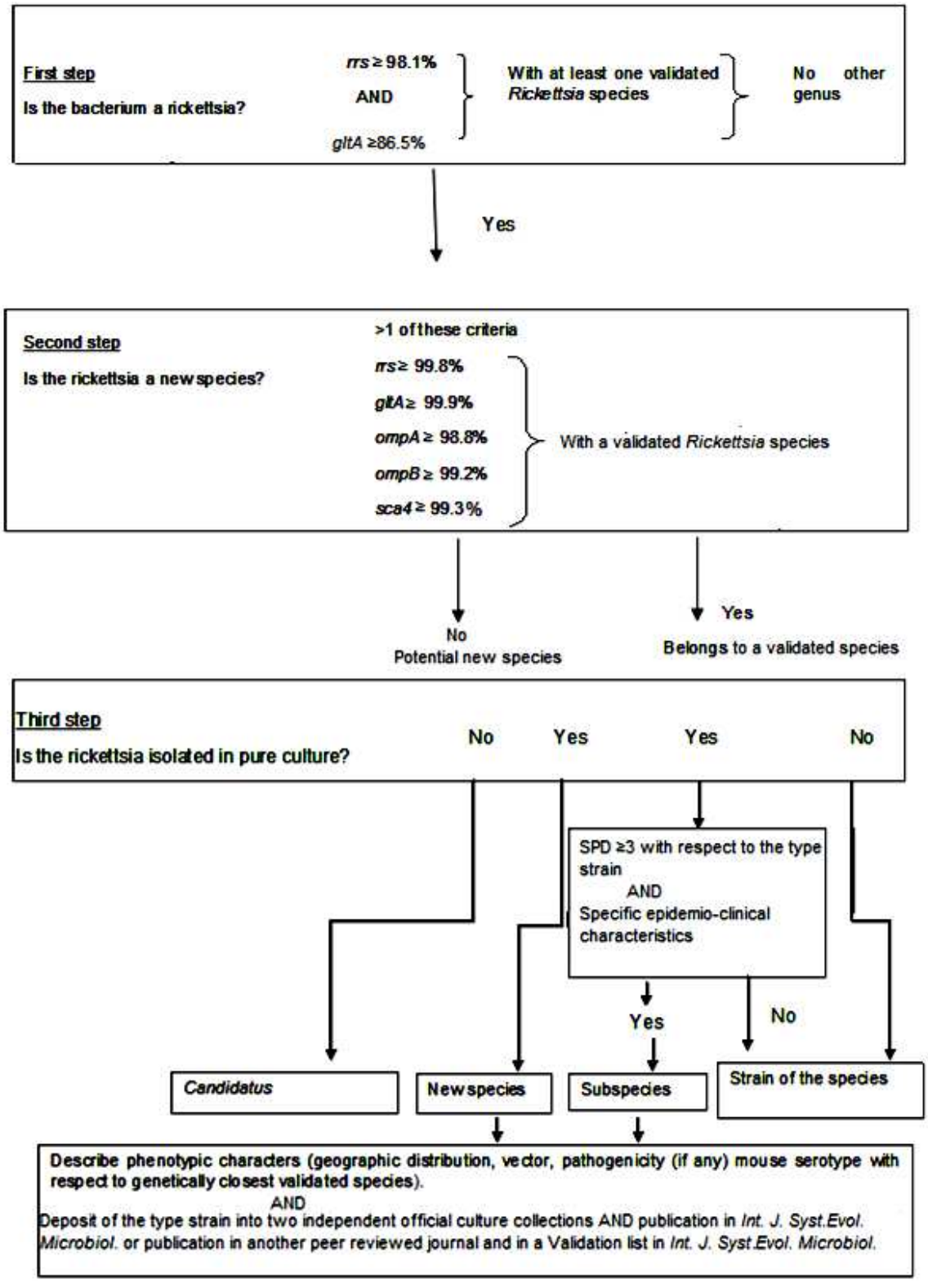


Figure 1.2: Guidelines for the classification of *Rickettsia* at genus, group, and species level using genotypic, phenotypic and phylogenetic characteristics. From Fournier *et al.,* (2009), licence number: 2878250837050.

1.2.2 The characteristics of the *Rickettsia* genome

Currently, thirteen rickettsial genomes have been sequenced completely and are available in the public domain (i.e. NCBI) (Anderson *et al*., 1998; Ogata *et al*., 2001, McLeod *et al*., 2004; Ogata *et al.,* 2005; Ogata *et al*., 2006). *Rickettsias* have circular genomes ranging in sizes from 1.1 to1.5 million base pairs (bp), with all the genomes having low GC content (approximately 30%). The genome contains approximately 1374 open reading frames (ORFs) and 150 palindromic elements interspersed between coding sequences (Blanc *et al.,* 2007). Comparisons of the published genome sequences revealed that *Rickettsia* comprises of 704 orthologous protein coding genes and 39 protein genes coding for RNA. From the 704 genes, 546 genes code for biochemical functions and the remaining 158 genes are poorly characterized and some of them code for genes that are associated with pathogenicity including the *ompA* gene and the type IV secretion system encoding genes (Blanc *et al*., 2007).

There are several regions within the rickettsial genome that are commonly used for identification and classification of *Rickettsia*. Those genes include the *gltA* gene (coding for the citrate synthase), antigenic proteins of Sca family (*ompA* and *ompB*) and the *16S rRNA* gene (Fournier and Raoult, 2009). Section 1.2.2.1-1.2.2.3 describes the characteristics of these genes.

1.2.2.1 The *16S rRNA* gene

The 16 subunit of the ribosomal RNA gene is approximately 1550 bp long and it consists of conserved and variable regions (Clarridge, 2004). This gene has been previously used to demonstrate that the genus *Rickettsia* belongs to the sub-order α-proteobacteria and has a common origin with *Erhlricia*, *Anaplasma* and *Wolbachia* (Brenner *et al.,* 2005; Fournier and Raoult, 2009). The sequences of this gene are conserved in the genus *Rickettsia*. It is the best tool for classification of *Rickettsia* to the genus and group level (Roux and Raoult, 1995; Fournier *et al.,* 2003, Fournier and Raoult, 2009). Several species within the genus *Rickettsia* share similar *16S rRNA* gene sequences (>98%) and therefore studies of this gene does not provide precise classification to the species level (Fournier and Raoult, 2009).

1.2.2.2 Citrate synthase (*gltA*) gene

Citrate synthase is a crucial component of almost all living cells and it is the enzyme of a central metabolic pathway, citric acid cycle which plays an important role in energy production and providing biosynthetic precursors (Roux *et al.,* 1997). Two main types have been identified; multimeric (found in all proteobacteria) and dimeric (found eukarya and Gram positive bacteria). The multimeric form is inhibited by NADH and 2-oxaloacetate and the dimer is inhibited by ATP (Roux *et al.,* 1997). The gene sequences of this enzyme are present in all *Rickettsia* (Regnery *et al.,* 1991; Eremeeva *et al*., 1994). The *gltA* sequence has higher diversity when compared the *16S rRNA* sequence and allows better discrimination of closely related *Rickettsia* species (Inokuma *et al*., 2001). This gene is approximately 1234 bp long and the "species–specific" sequences are recognized in this region (La Scola and Raoult, 1997; Fournier and Raoult, 2009).

1.2.2.3 Antigenic proteins (rompA and romp B)

Rickettsia is surrounded by crystalline proteins called the S-layer, which represents 10-15% of the total protein mass (Ching *et al.,* 1996). The S-layer is made up of immunodominant surface protein antigens (SPA). Two surface antigen proteins have been identified in the genus *Rickettsia* (rompA and rompB) and are the main antigenic determinants responsible for inducing the immune response in patients infected with *Rickettsia*. These proteins are involved in adhesion to the host cells (Ngwamidiba *et al.,* 2006).

The rompB protein is encoded by the *ompB* gene and it contains epitopes which are present in both SFG and TG *Rickettsia* (Roux and Raoult, 2000). It is the most abundant rickettsial protein and it contains genus, group and "species-specific"epitopes (Brenner *et al*., 2005). This gene is approximately 4776 bp long (Carl *et al.,* 1990) and the 5-end region of this gene exhibits higher variations (Parola *et al.,* 2005). This gene can be used to classify *Rickettsia* to the species level (Yu and Walker, 2006).

Rickettsial outer membrane protein A is encoded by the *ompA* gene. This gene is found in all SFG *Rickettsia* but not TG *Rickettsia* (Fournier *et al*., 1998; Yu and Walker, 2006). It is specifially found in SFG *Rickettsia* and displays an adequate heterogeneity to ensure accurate identification by comparisons of the 632 bp region at the 5' end of the gene (La-Scola and Raoult, 1997; Fournier *et al..* 1998; 2003). This gene can differentiate some strains of *R. conorii* and it demonstrates high

degree of interspecies diversity when compared to *gltA* and *16S rDNA* genes (Roux *et al*., 1996; Fournier *et al.,* 1998).

1.2.3 Pathogenesis

Members within the genus *Rickettsia* have been reported to cause human diseases around the world including Rocky Mountain spotted fever (RMSF), Mediterrenean spotted fever (MSF, also known as boutonneuse fever), murine typhus, epidemic typhus, rickettsial-pox, African tick bite fever (ATBF) and other spotted fevers (Walker, 2007; Yu and Walker, 2006; Fournier and Raoult, 2009).

1.2.3.1 Mode of transmission of *Rickettsia*

Members of the genus *Rickettsia* are transmitted to humans and other vertebrate hosts by arthropods such as ticks, fleas, lice and mites (Raoult and Roux, 1997) and therefore, their geographical distribution is determined by that of the host arthropod species (Azad and Beard, 1998). There are several routes in which *Rickettsia* can be transmitted to humans including saliva (during blood meal), feces (contamination of skin) and aerosols (dust containing infective fecal material) (Raoult and Roux, 1997; Kelly *et al*., 2004(b); Azad, 2007).

Ticks (ixodid or hard ticks) are the main reservoirs of SFG *Rickettsia*. *Rickettsia* infects and multiplies in almost every organ of the tick, including salivary glands. *Rickettsia* secreted in the tick's saliva is introduced to the host tissues as ticks insert their mouthparts into the host skin in search of a blood meal (Raoult and Roux, 1997; Parola *et al*., 2005; Socolovschi *et al*., 2009). These pathogens can be maintained transstadially and transovarily in ticks. Since all the developmental stages of the ticks are blood-feeding, the bacterium can also be transmitted to the different vertebrate hosts through blood-feeding (Brounqui *et al*., 2007). In mites as in ticks, *Rickettsia* is transovarilly maintained and is transmitted to susceptible host during a blood meal through the mite's saliva (Raoult and Roux, 1997).

Typhus *Rickettsia* (*R. prowazekii* and *R. typhi*) multiply in the epithelium of the intestinal tract of the arthropod vector and is excreted in feces. Transmission of the bacteria does not occur directly by bite but rather by contamination of the bite site by feces or the crushed bodies of infected lice (Raoult and Roux, 1997; Azad and Beard, 1998). The bacterium hematogenously spread in the body and ultimately invades the endothelial cells of the host (Raoult and Roux, 1997). Transmission to a

host can also occur through the respiratory tract by aerosols of dust containing infective fecal material (Kelly *et al*., 2004b).

1.2.3.2 Susceptible host

Members of the genus *Rickettsia* are obligate intracellular bacteria that require host cells to replicate and are associated with both invertebrates (arthropods; ticks, fleas, lice, mites, which act as their reservoir hosts) and vertebrates (mammals) (Raoult and Roux, 1997). Vertebrates are usually accidental hosts and *Rickettsia* is transmitted by infected arthropods (Raoult and Roux, 1997). In humans, rickettsial infection varies from mild (rickettsial-pox) to severe (Rocky Mountain spotted fever, epidemic typhus). In other animals rickettsial infection may present similar symptoms observed in humans e.g. in dogs during Rocky Mountain spotted fever (Varela, 2003), while other animals may remain asymptomatic, as in rats (*Rattus rattus*) during *R. typhi* or *R. akari* infection (Raoult and Roux, 1997).

1.2.3.3 *Rickettsia* infection cycle

Rickettsia is inoculated into the skin through the saliva of the infected tick, mite or flea. *Rickettsia*-laden feces can also be deposited by infected lice and fleas through scratching and can then spread throughout the body via the bloodstream to infect their target cells (Brenner *et al*., 2005). Interaction with the host involves three crucial steps; attachment, adhesion and escape from the phagosomal membrane (Yu *et al*., 2009). Outer membrane proteins (rompB and rompA) act as adhesions to the host cell. Rickettsial ompA and ompB bind to a transmembrane protein Ku70 in the host endothelial cell membrane and causes cytoskeletal actin rearrangements at the attachment site and this result in induced phagocytosis (Brenner*et al*., 2005; Walker, 2007; Yu *et al*., 2009). Once *Rickettsia* is phagocysed by the host cell, it rapidly escapes from the phagosome to the cytosol prior to phagosomal fusion avoiding exposure to lysosomal enzyme. In the cytosol *Rickettsia* acquire important nutrients such as glutamate as part of their energy requirement (ADP/ATP transporter) and amino acids for growth and replicates by means of binary fission (Walker *et al.,* 2003; Walker, 2007). Typhus group *Rickettsia* accumulates in massive quantities intracellularly and spread via bursting of massively infected host cells. Spotted fever group *Rickettsia* on the other hand, stimulate actin based mobility (cell to cell transmission) resulting in intracellular spread (Yu *et al*., 2009). Rickettsial infection leads to endothelial damage that is associated with vasculitis/inflammation of the blood vessels (Yu *et al*., 2009). Vasculitis occurs when heavily parasitized cells

cause cell proliferation and as a result a thrombus (blood clot) is formed (Yu and Walker, 2006). Thrombus interferes with the blood-flow and vascular permeability increases and causes an escape of *Rickettsia* in the red blood cells into the surrounding tissues. A lesion (eschar) develops when inflammatory cells flow into the tissue. Necrosis in the center of the lesion causes the representative clinical sign of rickettsial infection, the eschar (bite wound) (Walker, 2007).

The crucial pathophysiologic effect caused by rickettsial infection arises from increased vascular permeability with subsequent edema (abnormal accumulation of fluid beneath the skin), loss of blood volume, hypoalbuminemia (low albumin in the serum), decreased osmotic pressure and hypotension. These effects are all life threatening (Yu and Walker, 2006).

1.2.3.4 Clinical presentation of rickettsial disease

Athough clinical presentations differ with the causative agent, some frequent symptoms that usually develop within 5-10 days post-infection include fever, headaches, malaise and in some cases nausea and vomiting (Pretorius *et al*., 2004a). Most symptoms associated with acute rickettsial infections are unspecific and therefore further testing is required to make an accuarate diagnosis. Most tick transmitted rickettsioses are accompanied by rash or a lesion at the site of the tick bite (Pretorius *et al*., 2004a; Yu and Walker, 2006; Walker, 2007).

1.2.3.5 Immune response

Following infection with *Rickettsia*, host immune responses are rapidly triggered and contribute to the survival of host (Yu *et al.,* 2009). T-lymphocytes (CD8 and CD4 T-cells) and cytokines (IFN-γ and TNF-α and IL-1β) have been reported to play a more important role than antibodies in the immunodefense (Valbuena *et al.,* 2002; 2003). The CD4 and CD8 T-cells, macrophages and dentritic cells are recognized to be the sources of cytokines that activate endothelial cell rickettsiacidal activities (Walker, 2007). Through two bactericidal mechanisms, nitric oxide production and hydrogen peroxide production, endothelial cells are activated by IFN-γ and TNF-α and IL-1β kills intracellular *Rickettsia* (Valbuena *et al.,* 2002). Natural killer cells play an important role in the production of IFN-γ and clearance of *Rickettsia* within a few days of infection. Adaptive immunity (cell mediated immune response) is also important and appears to be long lasting and it is important in preventing re-infection rather than clearing primary infection (Walker, 2007). Antibodies against rickettsial ompB and ompA proteins are protective against re-infection with *Rickettsia* (Feng *et*

al., 2004). However, antibodies to these proteins do not appear until after the control of the rickettsial infection and recovery of the disease has occurred (Walker, 2007).

Figure 1.3 summurizes the indicators of rickettsial infection in relation to detection and diagnosis. The “Inoc phase” refers to the the time the arthropod vector inoculates the rickettsial agent in the host. “Eschar”- refers to the wound at the bite site that usually appears 1-2 days after an arthropod bite. “Fever” refers to the time the patient presents fever and other signs and symptoms of rickettsioses. “Rash”- refers to appearance of maculopapules usually 3-5 days after presentation of disease. IgM and IgG appears in detectable levels approximately at the same time (5-10 days) after onset of clinical symptoms. Culture, antigen and nucleic acid detection can occur prior to disease presentation if an eschar is apparent. Serological assays are performed 5-10 days post infection when IgM/IgG antibodies are detectable (Richards, 2011).

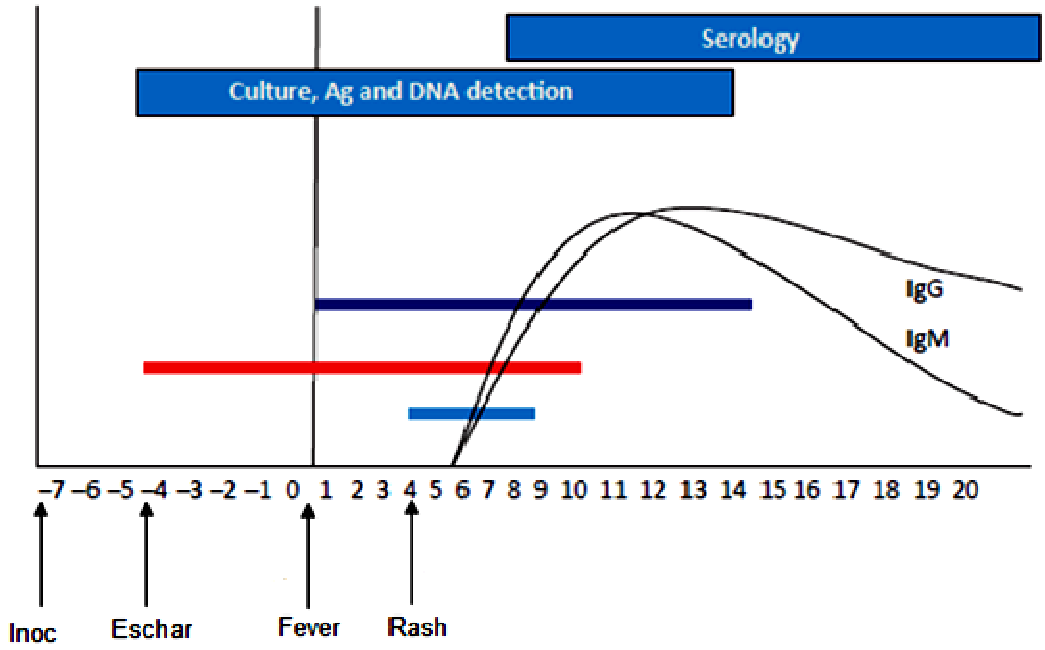


Figure 1.3: Time course of markers of rickettsial infection in association with detection and diagnosis. From Richards (2011), license number: 29044720303354

1.2.4 Diagnosis

Clinical examination and epidemiological history are the first steps to be considered when diagnosing a rickettsial infection. Clinical examination involves the symptoms of rickettsial infections and epidemiological investigation involves report of arthropod bite (tick, lice or flea) (Raoult and Roux, 1997). Several techniques that are frequently used for diagnosis of rickettsioses include classical staining (Gimenez staining), immunohistochemistry and paired serology (IFA, ELISA, and Weil Felix method) and/or nucleic acid detection methods (La-Scola and Raoult, 1997; Raoult and Roux, 1997; Parola *et al*., 2005).

1.2.4.1 Gimenez staining

The Gimenez stain is useful in detecting and identifying bacterial infections in tissue samples. It is valuable for detection of slow growing bacteria. *Rickettsia* does not stain well with the Gram stain, but are best characterized by Gimenez staining (La-Scola and Raoult, 1997; Yu and Walker, 2006). These organisms are extremely small (0.25 µm) and morphologically appear to be similar to the Gram negative bacteria but their cell walls have very thin peptidoglycan and lipopolysaccharide that weakly retain the counterstain safranin of the Gram stain (La-Scola and Raoult, 1997). With this technique, *Rickettsia* retains red stain with basic fuschin, while the background is decolorized and stains a purple-blue colour with the counterstain malachite green. Under the microscope *Rickettsia* appear as short rods (Gimenez, 1964; La-Scola and Raoult, 1997). This technique is not a common routine for diagnosis of rickettsial infection because it lacks sensitivity and specificity, but it is useful monitoring the presence of *Rickettsia* in cell culture (Kelly *et al.,* 2002; Richards, 2011).

1.2.4.2 Immunohistochemistry

Rickettsia can be confirmed in tissues by immunohistochemistry utilizing specific polyclonal or monoclonal antibodies against rickettsial proteins/antigens. Immunohistochemistry is often performed by using a bite wound biopsy specimen because concentrated rickettsial multiplications occur in this area (La-Scola and Raoult, 1997). This technique allows verification of rickettsial infection before sero-conversion and it permits early recommendation of a specific treatment (Raoult and Roux, 1997). Lepidi *et al.,* (2006) showed that this technique has a higher sensitivity than serology during the early stages of rickettsial infection. The disadvantage of this

technique is that it is labour-intensive, involves several procedural steps and cross-reactions among SFG *Rickettsia* has been reported (La Scola and Raoult, 1997; Takeda *et al.,* 2007).

1.2.4.3 Nucleic acid detection method

PCR and sequencing methods are now being used as sensitive and rapid tools to detect and identify *Rickettsia* in numerous specimens including blood, skin biopsies and arthropod tissues (Stein and Raoult, 1992; Williams *et al*., 1994, Brounqui *et al*., 1997; Raoult and Roux, 1997). PCR has an advantage over other techniques in that it avoids the use of culture, it is sensitive and specific and rickettsial DNA can be amplified directly from a clinical specimen (Raoult and Roux, 1997; Parola *et al*., 2005; Wölfel et *al*., 2008). In addition, *Rickettsia* species can be identified by direct sequencing of the reaction products. Several genes have been sequenced from *Rickettsia* isolates and as a result species description of various isolates and phylogenetic relationships between species have shifted rapidly. The criteria used for defining *Rickettsia* using gene sequencing has come under tremendous inspection (Fournier *et al*., 2003; Parola *et al*., 2005). However, recent studies by Fournier *et al*., (2009) revealed new criteria that can be used to classify *Rickettsia* to the species level. This study describes the use multi locus typing for classification of *Rickettsia* to the species level. Genes that are used include the *16S rRNA* (Roux and Raoult, 1995*)*, citrate sythase (*gltA*) (Raoult and Dasch, 1989), *ompA* (Raoult *et al*., 1996), *ompB* (Raoult and Dasch, 1989) and 17kDA protein encoding genes (Anderson and Tzianabos, 1989) (section 1.2.2).

The sensitivity of the conventional PCR using blood specimens can be potentially limited by low levels of circulating bacteria. In such cases additional steps such as the nested and heminested PCR are required to increase sensitivity. Real time quantitive PCR has also been shown to be a sensitive test for detection of *Rickettsia* even if present in low amounts in the specimen (Jiang *et al*., 2004; Kidd *et al.,* 2008; Wölfel *et al.,* 2008). This technique is not only sensitive but fast and it allows rapid quantification of the nucleic acid. It is less prone to contamination because the success of the reaction can be analyzed in real time. In addition to PCR, several nucleic acid detection methods have recently been developed to improve the detection of *Rickettsia* (Fluorescent in situ hybridization-FISH) and possibly identify *Rickettsia* to the strain level (Multi spacer typing-MST) (Fournier and Raoult, 2007; Svendsen *et al.,* 2009).

Multi-spacer typing (MST) involves the comparison of highly variable intergenic spacers for accurate typing of bacterial species (Fournier and Raoult, 2007). It has been used to classify of rickettsial isolates at the genus, species and strain level (Jado *et al*., 2006). It is based on the postulation that the non-coding sequences or the intergenic spacers are ideal for phylogenic analysis and assigning bacteria at strain level when compared to coding sequences that undergo less evolutionary pressure (Fournier and Raoult, 2007). This technique is based on polymorphism (gene variation) of intergenic spacers.

Fluorescent *in situ* hybridization (FISH) is a cytogenic technique used to identify and localize the presence or absence of specific DNA sequences (Svendsen *et al*., 2009). A fluorescent probe can only bind to parts of the genome with which they share a high degree of sequence similarity (Svendsen *et al*., 2009). Successful detection of *Coxiella* and *Bartonella spp* has been shown with this technique (Jensen *et al*., 2007; Gescher *et al*., 2008). It is simple, cost effective and well suited for detection of bacteria in the tissues of hosts. The application of FISH for detection of *Rickettsia* has been demonstrated (Svendsen *et al*., 2009). However, the probe used could only hybridize the SFG *Rickettsia*, although it was designed to detect all groups of *Rickettsia* (i.e. TG and SFG). It was concluded that FISH must be further evaluated using different genes in order to determine if it will be specific and sensitive for rickettsial diagnosis (Svendsen *et al*., 2009).

1.2.4.4 Serology

Serological techniques are the most commonly used and widely available methods to provide evidence of rickettsioses (Parola *et al.,* 2005; Richards, 2011). The antibody response to rickettsial infection is primarly directed against lipopolyssacharide (LPS) and outer membrane proteins (rompA and rompB) (Richards, 2011). Serologic assays that are currently used for the diagnosis of rickettsial diseases include indirect immunofluorescent assay (IFA), enzyme linked immunosorbant assays (ELISA) based on recombinant antigens, Weil Felix method and latex agglutination tests (Yu and Walker, 2006). For an acurate serological diagnosis, serum samples should be collected during the first, second, fourth and sixth week of illness. Typically, a serological test is accepted when patient has a minimum titer that is technique dependent (Evans and Branchan, 1998). The diagnostic titer may vary not only between laboratories but also in endemic areas for a particular disease (Yu and Walker, 2006). When rickettsial antigens represent the

agent of infection, titers of IgG or IgM antibody against this antigen should be at least two serial dilutions higher than titers of IgG/IgM antibodies against other rickettsial antigens (Parola *et al.,* 2005). IFA and ELISA assays are important for detection of IgM or IgG or both (Yu and Walker, 2006). IgM antibodies are usually detectable after the first week (5-10 days) of illness and may be elevated for an extended period (up to three months) and decline after antibiotic treatment. IgG antibodies peaks after the appearance of IgM usually after 7-14 days and may persist for months or years after acute infection, and tests that detect these immunoglobulins may be useful for sero-epidemiological studies (Gillepsie and Hawkey, 2006; Walker, 2007).

The indirect fluorescent assay is currently the gold standard technique and is used as a reference technique for the detection of *Rickettsia rickettsii* (agent of RMSF) and *R. typhii* (murine typhus) infections in most laboratories (La-Scola and Raoult, 1997; Yu and Walker, 2006; Richards, 2011). IFA is used to identify antibodies against *Rickettsia*. For an accurate diagnosis*,* serum specimens are taken in both early (acute) and later (recovery) stages of the disease. Specimens are collected 2-3 weeks apart in order to inspect a four-fold or greater increase in antibody titer. IgG antibodies are more specific and reliable and therefore, sera must be tested at least 7 days after the appearance of symptoms to ensure that seroconversion has occurred, because these antibodies only appear 7-10 days after the onset of the disease (La-Scola and Raoult, 1997; Yu and Walker, 2006). The IFA test for *Rickettsia* is normally group reactive rather than “species-specific”. The sensitivity of this technique ranges from 95-100% and specificity is 100% (Parija, 2009). The four-fold increase in antibody titers between the acute and convalescent (recovery) sera confirms diagnosis (Yu and Walker, 2006; Parija, 2009). The disadvantage of the commercially available IFA diagnostic kits is that cross-reaction between species within the same *Rickettsia* groups occurs (Sardelić *et al*., 2003; D Áuria *et al*., 2010).

There are two types of agglutination tests: Latex agglutination and the indirect hemaglutination test, however, the latex agglutination is most commonly used (Yu and Walker, 2006). In indirect hemaglutination tests, antibodies are detected against an antigenic erythrocytes-sensitizing substance (ESS). These antigens (ESS) are present in tissues and body fluids in the early stages of rickettsial infection, usually before a specific bacterial antibody is detectable (La-Scola and Raoult, 1997). ESS are obtained from SFG and TG *Rickettsia* by asn alkali extraction and adsorbed onto the sheep or human erythrocytes and these coated cells are used as antigens for

agglutination (La-Scola and Raoult, 1997). The ESS exhibits “group-specific” antigenic reactivity and cross reaction occurs among Rocky Mountain spotted fever, rickettsial pox, and Mediterranean spotted fever agents. This test can detect both IgM and IgG; however, agglutination is more efficient with IgM (Gillepse and Hawkey, 2006).

The latex agglutination test has been developed for diagnosis of Rocky Mountain spotted fever, Mediterranean spotted fever, murine typhus and epidemic typhus (Hechemy *et al*. 1981; La Scola and Raoult, 1997). In this technique, latex beads are coated with an ESS. The reactivity is higher when compared to indirect hemaglutination test because ESS on latex beads contains more antigenic fraction when compared to ESS adsorbed in erythrocytes. Latex agglutination reacts with both IgG and IgM antibodies. Antibodies to rickettsial infection can be detected within 1 week after the onset of illness (La-Scola and Raoult, 1997; Evans and Branchan, 1998; Yu and Walker, 2006). The assay is “group-specific” and sensitivity is equivalent with that of IFA. This technique is expensive but it is fast and does not require specialized equipments (Yu and Walker, 2006).

Available enzyme linked immmunoadsorbant (ELISA) methods focus upon specific reactivities to lipopolyssacharide (LPS), ompB and ompA antigens (Yu and Walker, 2006; Reeves *et al.,* 2006a; Liu, 2011, Richards, 2011). The lipopolyssacharide antigens have broad reactivity for spotted fever group *Rickettsia* and are commonly used for detection of IgG (Richards, 2011). The outer membrane antigens (rompB and rompA) are more “species-specific” and are often used for IgM testing. ELISA offers several major advantages over other serologic techniques in that they are easy to perform can be automated and do not require trained individuals for elucidation of results. This technique has low specificity when compared to other serologic assays and sometimes there is lack of standardization between laboratories (Gillespie and Hawkey, 2006). ELISAs are useful for surveillance studies, where a large number of sera can be assayed at the same time (Richards, 2011). One major reason ELISA cannot supplement IFA as gold standard for diagnosis of rickettsioses is that full length rompA and rompB antigens which are suitable for *Rickettsia* diagnosis are difficult to produce by recombinant methods and epitopes recognized by human and animal sera are present in many sites of these large proteins (Gillespie and Hawkey, 2006). However, recent studies reported that ELISA based on recombinant antigens (rompA, rompB and 56kDA lipoprotein

antigen) have higher sensitivities and specificities and allow early detection of antibodies and the cost of antigen production and maintenance is low (Jones and Barnard, 2007; Do *et al.,* 2009; Kowalczewska *et al.,* 2012).

The Weil Felix technique was previously used as the gold standard technique for diagnosing rickettsial infection in most developing countries (La-Scola and Raoult, 1997). This technique was recognized in the 1920s after it was observed that certain *Proteus* strains agglutinate with early-covalescent sera from patient with suspected rickettsial infection (Gillepsie and Hawkey, 2006). Epitopes of *Proteus vulgaris* OX2 agglutinate with sera of an individual infected with SFG *Rickettsia*, except *Rickettsia rickettsii* and *Rickettsia akari*. *Proteus vulgaris* OX19 epitopes react with the sera of an individual infected with typhus group *Rickettsia* and Rocky Mountain spotted fever (La Scola and Raoult, 1997). IgM antibodies are detectable in 5-10 days following the onset of clinical symptoms (Gillespie and Hawkey, 2006). This test has also shown that the test may be positive even though the levels of IgM are undetectable (La Scola and Raoult, 1997; Cowan, 2000; Jiang *et al*., 2004). The use of this technique is currently being discouraged in most countries including South Africa due to lack of sensintivity and specificity (Cowan, 2000; Frean *et al*., 2008; Rathi and Rathi, 2010). Other serological tests that have previously been used include Western blotting, complement fixation (CF) test and dot blot assays (La Scola and Raoult, 1997).

1.2.4.5 Isolation of *Rickettsia*

Isolation of *Rickettsia* from different specimens (clinical or ecological) requires stringent adherence to safety measures. Biosafety level 3 laboratories (for pathogenic strains e.g. *R. prowazekii*) and/or an approved safety cabinet in which negative air pressure is maintained are recommended (La Scola and Raoult, 1997; Parola, 2007). *Rickettsias* are generally difficult to isolate because of their obligatory intracellular lifestyles (associated with host cells). *Rickettsia* is most commonly isolated in cell culture, although historically isolation of *Rickettsia* employed the use of guinea pigs and embryonated eggs (Walker, 1996, La Scola and Rauolt, 1997) *Rickettsia* can be isolated in cell culture before the onset of clinical symptoms if inoculation eschar (lesion) is recognized but often successful isolation is achieved at the the time of disease presentation and for 1-2 weeks thereafter (Richards, 2011)

Animal models are often used if the samples are contaminated with other bacteria or if cell culture is not available (Gillespie and Hawkey, 2006). Guinea pigs are the animal of choice because they are susceptible to rickettsial infection (La Scola and Rauolt, 1997). Subsequent studies have shown successful isolation of *Rickettsia* in mice and rats (Raoult and Roux, 1997; Scheld *et al.,* 1999). Animals are inoculated with a suspension of tissue. Blood samples are collected from animals that show signs of illness post-inoculation. Target organs (e.g. spleen) are aseptically harvested and blood and tissue samples are repassaged in animals or embryonated eggs to increase the yield of the organism. Embryonated eggs can also be used for primary isolations (Raoult and Roux, 1997; Brenner *et al*., 2005). Rickettsial antibodies are tested in animals that survived 28 days post inoculations. Animals that were sero-negative before inoculation and are positive after 28 days are considered to be infected. Those that do not succumb to infection although sero-conversion has occurred, indicates infection with a strain of low virulence (Raoult and Roux, 1997).

Cell culture is currently the most commonly used technique for isolation of *Rickettsia* (Parola *et al*., 2005). Successful isolations of *Rickettsia* are performed from heparin anticoagulated plasma or buffy coat and skin biopsies collected at the bite wound. *Rickettsia* is cultivated in a wide variety of cell lines including the baby hamster kidney (BHK-2) cells, human erythroleukemia (HEL) cells and African green monkeys kidney epithelial cells (Vero) (Kelly *et al.,* 1991; Brenner *et al.,* 2005). Shell vial technique is commonly used to culture SFG and TG *Rickettsia* (Parola *et al.,* 2005). In this technique, a homogenized tissue or blood is inoculated onto the cell monolayers (Vero or BHK-2 or HEL cells) grown on coverslip in a shell vial tube and this is followed by low speed centrifugation and incubation at 35ºC in a CO_2 incubator. *Rickettsia* is detected directly inside the shell vial by immunofluorescence staining and microscopic examination and this technique is performed in triplicate. The medium is replaced after 48 hours. For detection of SFG *Rickettsia*, the culture is kept for two weeks and three weeks for examination of TG *Rickettsia*. After this time if immunoflourescence is negative, the culture is considered negative. When positive immunofluorescence results are obtained, parallel shell vials are inoculated into a confluent monolayer of suitable cell lines (Raoult and Roux, 1997; Parola *et al.,* 2005; Gillespie and Hawkey, 2006).

Successful isolation of *Rickettsia* has been shown with numerous monolayers of cells e.g. Vero, HeLa and BHK cell lines (Paddock *et al.,* 2006). In this technique, infected tissues are homogenized thoroughly in a sterile diluent and inoculated into monolayers of susceptible cells and incubated at 35° C in 5% CO_2 enriched atmosphere and monitored for growth. The medium is removed and replaced after 48 hours. The cultures are screened for infection with *Rickettsia* by using acridine orange stain, Gimenez stain or microfluorescence. The cultures are evaluated for 6-8 weeks before considered negative for *Rickettsia* (Paddock *et al*., 2006; 2008). Figure 1.4 summarizes the procedure followed when diagnosing rickettsial infections.

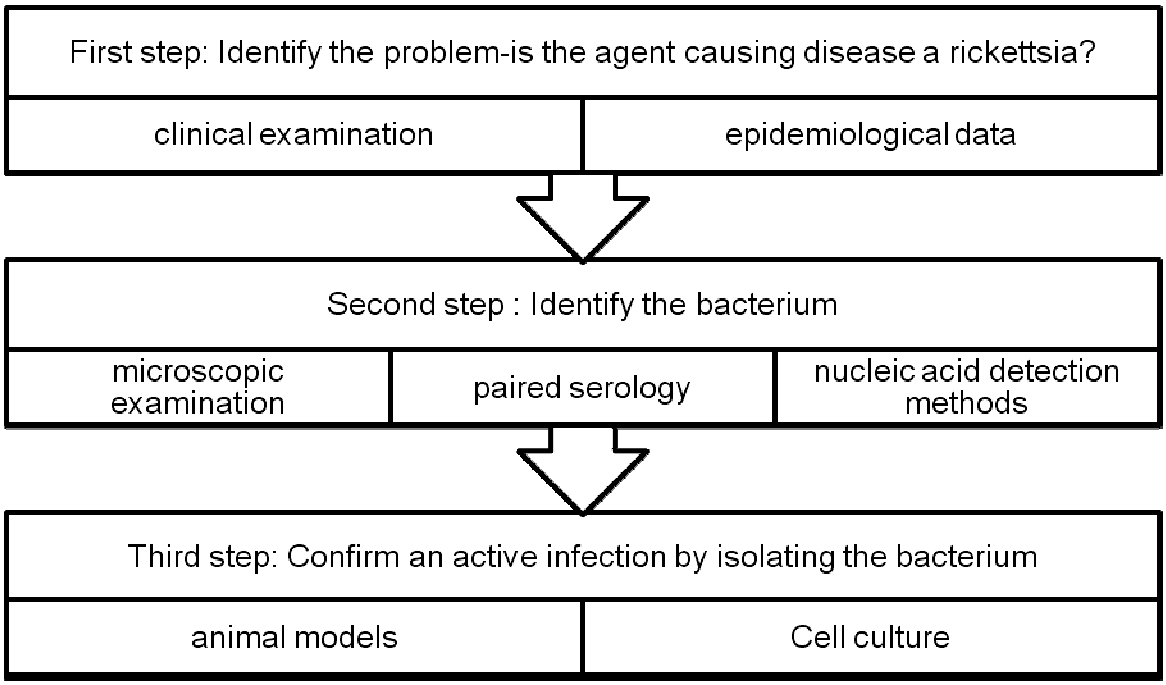


Figure 1.4: Guidelines used for diagnosis of rickettsial infections as described in Raoult *et al.,* (1997) and La Scola *et al.,* (1997)

1.2.4.6 Treatment

Diagnosing rickettsial infections may be difficult, but for rapid recovery, an early treatment with appropriate antibiotic therapy is fundamental (Pretorius *et al.,* 2004a; Frean *et al*., 2008). Treatment must be based on clinical suspicion and not be delayed awaiting the results of a laboratory test (Parola *et al.,* 2005). Chloroamphenicol was the first antibiotic used for treatment of Rocky Mountain spotted fever in 1948; however, the lethality of this disease was not completely reduced (Raoult and Drancourt, 1991). Side effects associated with the use of this

antibiotic include dose-related bone-marrow suppression (Pretorius *et al*., 2004a). Tetracycline was also used in the treatment of Rocky Mountain spotted fever, but had side effects such as permanent teeth discoloration in children and risks of inhibition of bone growth limited its usage (Pretorius *et al*., 2004a). The typical treatment routine of rickettsial diseases consist of 200mg of doxycycline daily for 3-14 days, but this antibiotic is not administered during pregnancy (Pretorius *et al*., 2004a; Parola *et al.,* 2005; Frean *et al*., 2008). The exact type and period of antibiotic treatment may differ depending on the disease and reduction of fever. Other antibiotics such as fluoroquinolones, rifampin and azithroycin may be considered, but are not approved for treatment because they have not been properly evaluated by controlled clinical trials (Raoult and Roux, 1997; Thorner *et al.,* 1998, Pretorius *et al.,* 2004a; Yu and Walker, 2006; Frean *et al*., 2008).

1.2.5 *Rickettsia* in Africa

About six rickettsial species have been reported in Africa (Hendershot and Sexton, 2009). Most surveillance studies for *Rickettsia* in Africa focuses on human infections and vectors responsible for transmission. Little has been reported on *Rickettsia* in other animals. Figure 1.5 summarizes rickettsial species that have been identified in different countries of Africa.

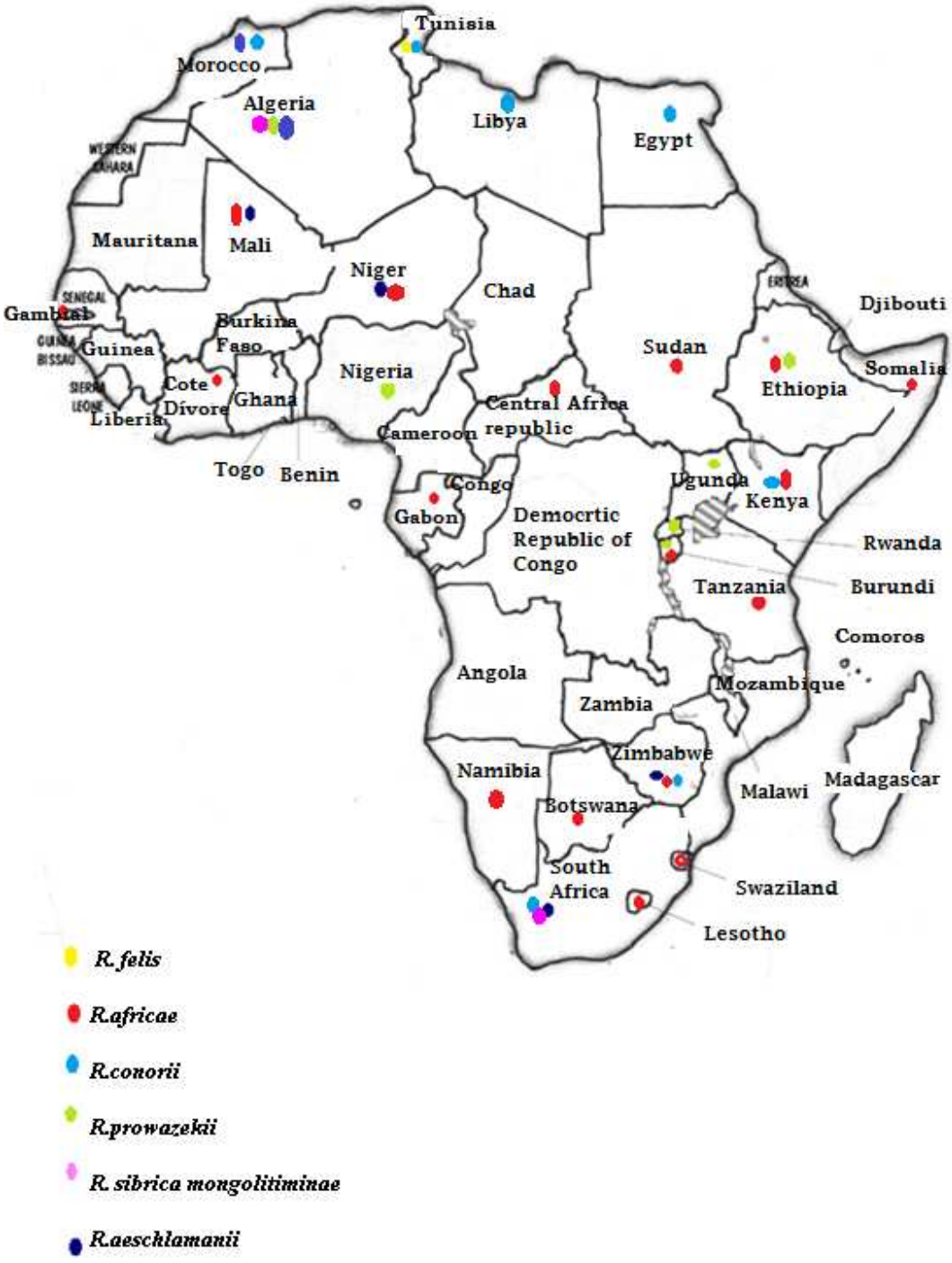


Figure 1.5: Map of Africa (From Mongab.com/date accesed: 23-09-2011) showing the known distribution of rickettsial species in Africa. *Rickettsia* species are indicated by the coloured dots. The location of the dots does not indicate the exact area in which the rickettsial species was identified, it indicates only the country.

1.2.5.1 *Rickettsia africae*

Rickettsia africae is the known agent of African tick bite fever (AFBF), and it is the most common rickettsial-borne disease in Sub-Saharan Africa (Kelly and Mason, 1990). *Amblyomma hebraeum* are the vectors of *R. africae* in southern African countries and *A. variegatum* are vectors of this agent in west and central Africa (Parola and Rauolt, 2001). These ticks infest ruminants and wild animals and can aggressively attack humans (Hendershot and Sexton, 2009). The geographical distribution of African tick bite fever appears to correspond to that of *Ambylomm*a spp. *Rickettsia africae* is transtadially and transovarily maintained in *A. hebraeum*; therefore all feeding stages can transmit infection to susceptible hosts. This tick is common in rural areas (Parry, 2004). Rapid expansion of safari tourisms has led to the re-emergence of *R. africae* in areas where it was not endemic. AFBF is common in soldiers, game hunters and individuals who have been exposed to wild animals (Jensenius *et al*., 2003). It was first described in the 1930s by Pijper (Pijper, 1934, Pijper, 1936) and was properly named in the 1990s after serotyping rickettsial isolates from *Ambylomma hebraeum,* and it was found that they exhibited milder symptoms when compared to that of *R. conorii* (Kelly *et al.,* 1991). African tick bite fever is rarely associated with cutaneous rashes (Caruso *et al*., 2002). This disease has been reported in south, east and west and central Africa (Hendershot and Sexton, 2009) (Figure 1.5). In South Africa, it is commonly recognized in tourists with an incidence rates of infection of 4–5% in European tourists (Roch e*t al.,* 2008; Frean *et al.,* 2008; Hendershot and Sexton, 2009; Althaus *et al.,* 2010).

1.2.5.2 *Rickettsia conorii*

Rickettsia conorii is an agent of Mediterranean spotted fever (also known as boutenneuse fever) and it is transmitted to humans by dog ticks, *Rhipicephalus sanguineus* (Brounqui *et al*.., 2007). These ticks are well adapted in urban areas (Hendershot and Sexton, 2009) and can bite humans in the absence of a primary host. Numerous cases of Mediterranean spotted fever have been reported in Morocco, Libya and Egypt (Parola *and* Rauolt, 2001; Hendershot and Sexton, 2009). MSF was first described in Tunisia by Conor in 1910 (Parola *et al.,* 2005), where it is now considered as the predominant rickettsial disease (Kaabia *et al.,* 2006; Parola, 2006). This bacterium was recently also detected in Senegal (Mediannikov *et al.,* 2010) and is common in southern Africa and causes a more severe tick bite fever called boutenneuse fever tick-bite fever (Frean *et al*., 2008).

1.2.5.3 *Rickettsia prowazekii*

This bacterium is transmitted to humans by a human body louse, *Pediculus humanus humanus,* and it is an agent of epidemic typhus*. Pediculus humanus humanus* is distributed worldwide and infests on humans (Parola and Rauolt, 2001). These lice can survive without the human host but they die of starvation 10 days after removal (Hendershot and Sexton, 2009). It is common in refuge populations were body and head lice are prevalent. The first outbreak of typhus occurred during World War I and II in the Pacific Islands and Europe and throughout North Africa (Parola, 2006). Approximately 1,931 cases were reported in Ethiopia in 1983 and 1984 (Raoult *et al.,* 1998). Epidemic typhus is a potential threat to travelers to Burundi, Nigeria, Rwanda and Ethiopia (Parola *et al*., 2005; Hernershot and Sexton, 2009). Currently there is no study that has reported epidemic typhus in South Africa.

1.2.5.4 *Rickettsia sibirica subsp. mongolotimonae*

*Rickettsia sibirica mongolotimonae (*formerly known as *Rickettsia mongolotimonae)* was first identified in *Hyalomma asiaticum* (hard tick that infest on domestic animals) in Mongolia in 1991 (Brounqui *et al.,* 2007). The name *R. mongolotimonae* was given to this *Rickettsia* based on different sources of isolates (thus Mongolia and La timonae hospital in Marsilae). Based on gene sequence criteria the bacterium was identified as a member of *R. sibirica,* but phylogenetically the strains of *R. mongolotimonae* clustered separately within the *R. sibirica* strains. The serological and ecological characteristics of *R. mongolitimonae* were different from the *R. sibirica* complex. Based on Latin nomenclature, it is now called *R. sibirica subsp. mongolotimonae* (Fournier *et al.,* 2003; Parola *et al.,* 2005). *Rickettsia mongolotimonae* was detected in sub-Saharan Africa in 2001, in *Hyalomma truncatum* ticks*.* This bacterium parasitize on migratory birds that are distributed in several African countries including South Africa. It was identified in Ellisras (Limpopo province: South Africa): when a construction worker suffered from severe headaches and fever and had an eschar at the site of a tick bite. After several molecular studies (PCR) and serological tests it was confirmed to be a *Rickettsia sibirica mongolotimonae* infection (Pretorius *et al.,* 2004b).

1.2.5.5 *Rickettsia aeschlimannii*

This bacterium was first isolated in *Hyalomma marginatum* (hard tick that parasitize on domestic animals including cattle, sheep, birds and pigs) collected in Morocco in 1997 (Bearti *et al*., 1997). Subsequent isolations of the same bacterium in these ticks were reported in Mali, Niger and Zimbabwe (Parola and Rauolt, 2001). The first human case was in Morocco in 2000 (Raoult *et al*., 2002). It was detected in a South African man returning from hunting and fishing trips. The man was bitten by a *Rhipicephalus appendiculatus* tick and an inocualtion eschar was evident around the tick's attachment site. After molecular studies and serologic analysis of blood and tissue specimens, it was confirmed as a *R. aeschlimanni* infection (Pretorius and Birtles, 2002).

1.2.5.6 *Rickettsia felis*

Rickettsia felis was first detected in 1990 in a cat flea, *Ctenocephalides felis,* and it was named ELB agent after the Elward laboratory (Soqeul, CA) were the flea colony was raised (Fournier *et al.,* 2008). It is transmitted to humans and other susceptiple hosts by *Ctenocephalides felis* fleas which also act as the reservoir host. Infection with this bacterium results in murine typhus. This rickettsial species enters the body through a bite wound or contamination of the bite site by feces deposited by an infected flea. It has been reported in numerous flea populations in North and South America (Perez-Osoria *et al*., 2008) and it has been described in humans in Tunisia (Znazen *et al*., 2006). It has been detected in the Democratic Republic of Congo in the flea; *Pulex irritans* (Sackal *et al*., 2008).

1.2.6 Bats and rickettsial species

Rickettsia have been reported in a wide range of hosts including rodents (Choi and Lee, 1996), blood sucking arthropod vectors (Raoult and Roux, 1997;Parola *et al.,* 2005), felines (Kitcharoen *et al.,* 2010), livestock (Wendincamp *et al*., 2002) and humans (Hendershot and Sexton, 2009). There is no study to date that has investigated the prevalence of *Rickettsia* in bats in Africa. Few studies have reported sero-positivity against rickettsial antigens in bats (Reeves *et al.,* 2006a; D Áuria *et al*., 2010). In the first study, big brown bats *Eptesicus fuscus*, were screened for antibodies against *Rickettsia, Borriella,* and *Bartonella* in Georgia; USA. Sero-conversion against *Rickettsia rickettsii* antigen was detected in 1.8% (1/56) of the bats tested, but no rickettsial DNA was detected (Reeves *et al*., 2006a). In another study in Brazil, bats from three different families *(Molossidae, Vespertilionidae* and

Phyllostomidae) were screened for antibodies against rickettsial antigens. Overall sero-conversion was 8.6% (39/451), 9.5% (34/358), 7.8% (28/358) and 1.1% (4/358) agains*t R. rickettsii, R. parkeri*, *R. amblyommi* and *R. rhipicephali*, respectively (D Áuria *et al*., 2010). Unlike in Reeves *et al.,* (2006a), they did not attempt to detect rickettsial DNA.

1.2.7 *Rickettsia* in bat ectoparasites

Bats carry a wide range of ectoparasites including flies (*Nycteibiidae* and *Streblidae*), fleas (*Siphonaptera*), mites (*Spinturnicida*e and *Maccrosynidae*) chingers *(Thrombociludae*) and ticks (*Argasidae*) and many of which are highly "host-specific" and die when they are dislodged from their hosts (Dick *et al*., 2006). To date, there are two publications that have reported *Rickettsia* in bat ectoparasites. Several rickettsial pathogens were detected in mites (*Spinturnix* and *Steatonyssu*s) collected from *Miniopterus spp*. from Tanzania and Madacascar (Reeves *et al*., 2006b). In another study, rickettsial DNA was detected in 28/31 live and 60/90 dead *Carios kelleyi* ticks collected from community buildings in Jackson County; Iowa (USA). In addition, 5 female ticks that tested positive for *Rickettsia* laid eggs that also tested positive for rickettsial DNA indicating a possible transovarial transmission (Loftis *et al.,* 2005). The *Carios kelleyi* tick is an argasid tick (soft tick) that is found on bats and in their habitats and can feed on humans in the absence of the primary host (Gill *et al*., 2004).

1.2.8 Possible transmission of *Rickettsia* from bats to humans

Transmission of *Rickettsia* to humans by bats or bat ectoparasites has never been reported. Reeves *et al.,* (2006a) investigated the potential health risks of big brown bats (*Eptesicus fuscus)* to humans, since they reside closer to human dwellings and carry ectoparasites that can feed on humans. In this study, the *Carios kelleyii* ticks (parasites of *E. fuscus)* that were naturally infected with spotted fever group *Rickettsia* were allowed to feed on pathogen free guinea pigs (*Cavia porcellus*). Blood was collected on day 0, 3, 7, 14, 21 and 35 for PCR detection of rickettsial DNA and serological assays. The guinea pigs were euthanized after 35 days and organs were collected and PCR was performed on the DNA extracted from the organs. A weak sero-conversion (1:32) to rickettsial antigens was detected 14 days post-infestation but the titer remained the same until day 35 and the rickettsial DNA was not detected. The data showed that only a small quantity of the rickettsial antigen was inoculated by the larval ticks and as a result it did not infect the guinea

pigs. However, it does not refute the possible transmission of rickettsial pathogens to other hosts by these ticks during blood meal. There is no study that has implicated transmission of *Rickettsia* to bats by ectoparasites (Pearce and O'Shea, 2007).

Destruction of the natural habitats of bats as a result of urban expansion, increased tourisms to caves and climate changes has resulted in a closer association of bats with humans (Wong *et al.,* 2007). These associations might pose health risks to humans. Numerous studies have reported that bats carry vector borne bacteria and ectoparasites that can bite and cause diseases in humans (Ghatak *et al*., 2000; Gill *et al.,* 2004; Wong *et al.,* 2007; Evans *et al.,* 2009; Childs-Sandfords *et al.,* 2009; Bai *et al*., 2011). In addition, bats' feces have been reported to carry important pathogens that can cause human infections (Wong *et al.,* 2007).

1.3 *Bartonella*

Members of the genus *Bartonella* are small (approximately 0.3µm in size), Gram negative bacilli, and aerobic, facultative intracellular pathogens that are associated with erythrocytes and endothelial cells of mammals and other vertebrates (Anderson and Neumann, 1997). *Bartonella* are transmitted between the mammalian hosts by arthropod vectors such as bitting flies, lice, fleas, sand flies and ticks (Boulouis *et al*., 2005, Chomel *et al*., 2009). Currently, the genus *Bartonella* consist of 25 validated species and 13 of these species cause human infections (Chomel and Karsten, 2010).

1.3.1 *Bartonella* taxonomy and phylogeny

Only two diseases caused by *Bartonella* species were recognized in 1990: Carrión's disease (caused by *B. bacilliformis*) and trench fever (caused by *B. quintana*) (Karem, 2000). *B. bacilliformis* was first recognized in 1909 by Alberto-Barton in the erythrocytes of a patient suffering from Oroya fever, which is an acute form of Carrión's disease (Ihler, 1996). The genus *Bartonella* was merged with the genus *Rochalimaea* based on DNA-DNA hybridisation information and comparisons of *16S rRNA* gene sequences following the proposal by Brenner *et al.,* (1993). The genus *Rochalimaea* comprised of 3 species: *Rochalimaea vinsonii;* which was first isolated in small rodents (Barker, 1946), *Rochalimaea quintana,* which is an agent of trench fever and isolated in 1918 (Strong, 1918) and *Rochalimaea henselae* first described in 1992 and the agent of cat scratch disease. *Rochalimaea* species are now called *Bartonella vinsonii*, *Bartonella quintana* and *Bartonella henselae,* respectively.

Furthermore, the genus *Grahamella* was merged with the genus *Bartonella,* on the basis of phylogeny following a proposal by Birtles *et al*., (1995). The species within the genus *Rochalimaea* and *Grahamella* are now unified and fall under the genus *Bartonella*. Phylogenetically the genus *Bartonella* belongs under the α2-subgroup proteobacteria, in the order *Rhizobiales,* and the family *Bartonellaceae* and is closely related to the genus *Brucella, Agrobacterium* and *Rhizobium.* Historically, *Bartonella* species were incorporated in the family *Rickettsiaceae* and the tribe *Rickettsieae*, which was subdivided into three genera: *Rickettsia*, *Coxiella*, and *Rochalimaea* (Brenner *et al.,* 2005*).* The introduction of molecular techniques allowed proper estimation of phylogenetic relationships of bacteria. Analysis of the *16S rRNA* gene sequences led to the removal of the genus *Coxiella* and *Rochalimaea* from the *Rickettsiaceae* family and renaming of the genus *Rochalimaea* and *Grahamella* to *Bartonella* (Birtles *et al.*, 1995; Clarridge *et al.*, 1995; Anderson and Neuman, 1997; Greub and Raoult, 2003). Figure 1.6 indicates bacterial species under the genus *Bartonella*.

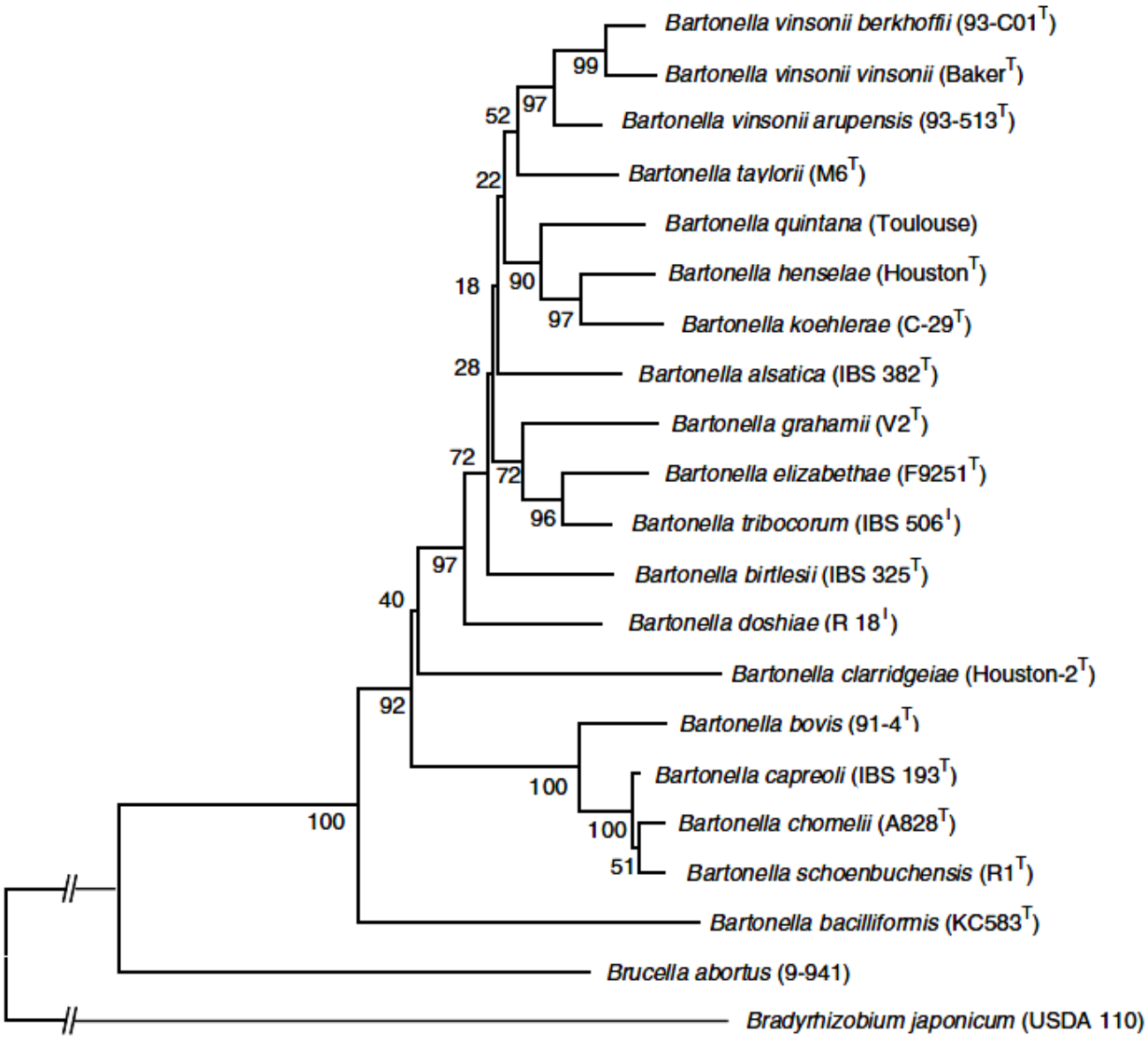


Figure 1.6: Phylogenetic analysis of the genus *Bartonella* on multilocus sequence analysis. From Saenz *et al.,* (2007), licence number: 2878761140434.

Defining a new species of the genus *Bartonella* was based on DNA-DNA hybridizations and phenotypic traits. These methods had major drawbacks such as the practicality and difficulty of performing DNA-DNA hybridizations and the absence of distinguishing phenotypic traits (La-Scola *et al.,* 2003). Several genes or intergenic regions are now being suggested to be used for defining new species of the genus *Bartonella*. This include the citrate synthase gene (*gltA)*, cell replication gene (*ftsZ)*, riboflavin synthase gene (*ribC*), *16S rRNA*, *16S/23S* intergenic spacer, the heat shock protein encoding gene *(groEL)* and the RNA polymerase subunit encoding gene (*rpoB*) (La-Scola and Raoult, 2003). Currently, other genera such as *Rickettsia*

are also using this approach (multi locus sequencing) to define organisms up to the species level (Fouriner and Raoult, 2009). Table 1.1 summarizes the cut-off sequence similarity values used for species definition as described in La Scola *et al*., (2003).

Table 1.1: Cut-off sequence similarity values of genes used for species definition in the genus *Bartonella.* From La Scola *et al.,* (2003), licence number: 2880670916315.

Gene	**Percentage of gene similarity**		
	Median value	Highest value	Lowest value
16S rRNA	99.7	99.8	98.3
ITS	93.9	99.8	89.2
gltA	93.6	96.0	87.1
GroEL	92.6	99.4	86.2
RpoB	92.8	95.4	90.9
FtsZ	94.4	97.9	88.8
RibC	86.5	99.5	82.8

1.3.2 Characteristics of the genome

Bartonella have circular genomes ranging from 1.44 (coding for 1,283 genes) to 2.62 (coding for 2,136 genes) million base pairs. *Bartonella* share approximately 959 genes that are characteristics of host integrated metabolism (Engel and Dehio, 2009). The genome has a GC content of approximately 38.5-41.1 % (Brenner *et al.,* 1991; Daly *et al*., 1993). Phylogenetic relationships are usually determined using the *16S rRNA* gene (Zeaiter *et al.,* 2002), the *gltA* gene (Birtles and Raoult, 1997), *FtsZ* gene (Zeaiter *et al.,* 2002), *ribC* gene *(*Zeaiter *et al.,* 2002), and the *groEL* genes (Swegger *et al*., 2000). Section 1.3.2.1-1.3.2.5 describes the characteristics of these genes.

1.3.2.1 The *16S rRNA* gene

The *16S rRNA* gene is approximately 1500 bp long and has played a major role in determining the the phylogenetic position of the genus *Bartonella* among proteobacteria (La Scola *et al.,* 2003; Brenner *et al*., 2005). It is useful for phylogenetic studies of *Bartonella* at the genus level (Zeaiter *et al*., 2002; Brenner *et al.*, 2005). It is not a good candidate gene for identification of genetic variability

among *Bartonella* species, because the levels of sequence similarities are high (97-99%) (Zeaiter *et al.,* 2002; La Scola *et al.,* 2003).

1.3.2.2 The citrate synthase gene (*gltA*)

Citrate synthase is the first enzyme of the citric acid cycle and it is the main regulator of intracellular ATP in both eukaryotes and prokaryotes. The gene-sequences of this enzyme are highly conserved in all proteobacteria (both α and λ) (Fournier *et al*., 2003). The citrate synthase gene is approximately 1239 bp long and it is useful for phylogenic studies of *Bartonella* to the species level (Drancourt *et al.,* 1996). Studies have shown that the *gltA* gene can be used to discriminate closely related *Bartonella* species, not strains (Birtles and Raoult, 1997; Zeaiter *et al.,* 2002). Comparisons of the *gltA* sequences of different *Bartonella* species revealed that levels of similarities were 83.8-93% whereas comparisons of sequences obtained from different strains of the same species revealed that levels of similarities were more than 98% (Zeaiter *et al.,* 2002).

1.3.2.3 The cell division protein encoding gene (*FtsZ)*

The FtsZ protein plays a key role in bacterial cell division and it is larger in *Bartonella* when compared to other bacteria because it has an additional C-terminus end (Kelly *et al.,* 1998; Ehrenborg *et al*., 2000; Zeaiter *et al.,* 2002). The gene encoding for this protein has been used to identify *Bartonella* to the species level. The 900 bp nucleotide sequence of the *FtsZ* encoding N-terminal region is useful in differentiating closely related *Bartonella* species and the 880 bp nucleotide sequence of the *FtsZ* C-terminal region is important for sub-typing closely related *Bartonella* species (Zeaiter *et al.,* 2002).

1.3.2.4 The heat shock protein encoding gene (*groEL*)

The groEL protein is one of the two highly conserved components of the heat shock chaperone response system and it is important for proper folding and assembly of polypeptides (Zeaiter *et al*., 2002). The gene encoding the groEL protein is approximately 1368 bp long and has been used to infer intraspecies phylogenetic relationships and subtyping *Bartonella* species (Zeaiter *et al*., 2002).

1.3.2.5 The riboflavin synthase gene (*ribC*)

Genes encoding enzymes of the riboflavin biosynthetic pathway are conserved in bacteria and plants but are absent in humans. Therefore, this makes them an excellent candidate for the detection and discrimination of pathogenic bacteria (Johnson *et al.,* 2003). The *ribC* gene is approximately 821 bp long and has successfully been used to discriminate closely related *Bartonella* species and sometimes strains (Bereswill *et al*., 1999; Bacher *et al.,* 2001; Johnson *et al.,* 2003).

1.3.3 Pathogenesis

Members of the genus *Bartonella* have been reported to cause human diseases world-wide including cat scratch disease (CSD), Oroya fever, trench fever, bacillary angiomatosis (proliferation of blood vessels), cardiac disease and endocarditis (inflammation of the heart muscles) (Anderson and Neuwmann, 1997; Breitschwerrdt and Kordick, 2000; Chomel *et al.,* 2009).

1.3.3.1 Mode of transmission of *Bartonella*

Bartonella are transmitted to humans and other vertebrate hosts by blood-sucking arthropods such as ticks, fleas, lice and biting flies or through contamination of a bite wound by infected fecal material deposited by infected flea or lice (Breitschwerdt and Kordick, 2000). *Bartonella* can occupy the mammalian erythrocytes and therefore has the probability to infect a variety of arthropod vectors during a blood meal (Valentine *et al.,* 2007). Transmission of *Bartonella* to other hosts can occur in two ways: saliva (during feeding) and by fecal material (contamination of skin) (Breitschwerdt and Kordick, 2000; Bilieter *et al.,* 2008).

1.3.3.2 Susceptible host

Bartonella spp. have been detected in wide range of hosts including felines, rodents, canines and humans (Kosoy, 2009; Chomel and Kasten, 2010). Cats represent the largest reservoir of *Bartonella* spp. responsible for human infections (Chomel *et al*., 2009). Transmissions to humans occur via direct scratches or a bite from an infected cat. Cats are the main reservoirs of *B. henselae, B. clarridgeiae* and *B. koehlerae* (Breitschwerdt and Kordick, 2000; Chomel and Kasten, 2010; Breitschwerdt *et al.,* 2010). Other species that were also isolated from cats include *B. quintana, B. bovis* and *B. vinsonii subsp berkoffii* (Chomel and Kasten, 2010; Breitschwerdt *et al*., 2010*). Bartonella henselae*, *B. clarridgeiae* and *B. koehlerae* are transmited among

cats by fleas. The extent to which these species are pathogenic to cats remain unclear. Recent studies have shown that cats can develop endocarditis, kidney disease, urinary tract infections and lymphadenopathy (swollen lymph nodes) due to *B. henselae* infection (Chomel and Kasten, 2010; Breitschwerdt *et al*., 2010).

Bartonella spp. that have been reported in dogs include *B. vinsonii berkofii, B. elizabethae, B. rochalimaea, B. clarridgeiae* and *B. quintana* (Kernif *et al.,* 2010; Breitswercht *et al.,* 2010). Transmission of *Bartonella* species from dogs to humans can occur through an arthropod vector or alternatively by a dog scratch or bite (Duncan *et al*., 2007). Studies have shown that dogs infected with *Bartonella* species may develop disease symptoms that are similar to those reported in humans, for example endocarditis (inflammation of heart muscles) and lymphadenitis (swollen lymph nodes) (Breitschwerdt *et al*., 1996; 1999; Kernif *et al.,* 2010).

Rodents serve as a natural reservoir for numerous *Bartonella* species including *B. grahamii*, *B. vinsonni subsp aurepensis, B. taylorii* and *B. elizabethae* (Boulouis *et al*., 2005). There is limited data reporting human infections caused by rodent-borne *Bartonella* (Boulouis *et al*., 2005). Among rodent-borne *Bartonella, B. elizabathae* has been reported in injection drug users and homeless people who have been exposed to rats, their ectoparasites (mites) and fecal material (Comer *et al.,* 1996).

Bartonella species are important human pathogens (Dwornick *et al.,* 2006). There are about 25 species of *Bartonella* identified to date and 13 of these species have been associated with human diseases (Chomel *and* Kasten*,* 2010; Paziewska *et al*., 2010). Those species include; *B. bacilliformis* causing Oroya fever and verruga peruana fever, *B. henselae* causing cat scratch disease, *B. quintana* causing trench fever and endocarditis, *B. clarridgeiae* causing cat scratch disease, B. *vinsonii susp aurupenis* causing persistant bacteremia (bacteria in the blood), *B. grahamii* causing neuroretinitis (swelling of the optic disc) and *B. washoensis* causing cardiac disease (Dworkin *et al.,* 2006). Other species include: *B. tamiae*, *B. elizebethae, B. koehlerae*, *B. vinsonni berkoffii*, *B. talpae* and *B. alsatica* (Chomel and Karsten, 2010).

1.3.3.3 *Bartonella* infection cycle

Infections with *Bartonella* begin with inoculations of bacteria either during a blood meal by an infected arthropod through saliva or through contamination of skin by infected fecal material deposited by an infected flea or louse (Jacomo *et al*., 2002). Immediately after infection, *Bartonella* colonizes in the endothelial cells and every 5 days *Bartonella* are released from the endothelial cells into the bloodstream where they infect the erythrocytes. *Bartonella* invade and replicate in the phagosomal membrane inside the erythrocytes and multiplies until critical density is reached (Angelakis *et al*., 2010). The symptoms of *Bartonella* infections vary with different species, but generally appear 10-15 days post-infection (Angelakis *et al*., 2010).

Bacterial adhesions (autotransporter adhesins) mediate the important step early in pathogenesis of *Bartonella* (Zhang *et al.,* 2004). Autotransporter adhesions bind to the external matrix machinery of the host leading to a firm bacterial adhesion to the cell surface and this ensures efficient translocation of the type IV secretion systems. Type IV secretion systems are associated with bacterial conjugation and assist in the adaptations of *Bartonella* to the mammalian hosts (Dehio, 2001; 2004). Differences in clinical symptoms of individuals during *Bartonella* infection rely on several factors such as the amount of the inoculum and genetic variations in the strain. These factors are related to the difference in the severity of the clinical signs that may occur as well as the intensity of the illness (Jacomo *et al.,* 2002).

1.3.3.4 Immune response

The pathological response to *Bartonella* infection varies with the status of the host immune response. For example cat scratch disease can cause a minor reaction in an immunocompetent patient (formation of pus), but might result in a more severe manifestation bacillary angiomatosis (proliferation of blood vessels) in immunocompromised individuals (Maurin and Raoult, 1996). Both humoral immune response (B-lymphocytes; antibody production) and cell mediated immunity (T-lymphocytes) are important. Humoral immune responses differ between hosts. For example, reservoir hosts such as cats can build up a significant humoral response even though bacteremia (bacteria in the blood) persists. In other hosts such as small mammals, antibodies are barely detected despite high prevalence of bacteremia (Kosoy *et al.,* 1997; 2004). Cellular mediated immunity is the most frequent mechanism employed by the host to many intracellular bacteria (Schaible *et al*., 1999). In cellular mediated immunity, the major histocompatibility complex

(MHC) plays an important role in presenting antigens to the CD8+ T cells (Pederson *et al*., 1998). However, mammals' erythrocytes do not have MHC of the surfaces. Thus, despite the existence of *Bartonella* in the erythrocytes, the cellular mediated immune response may not be triggered leading to chronic *Bartonella* infections (Dehio, 2001). Several studies have shown that in some cases, the host immune defenses may be improved by activation of CD4+ T-helper cells (Th1 and Th 2) (Karem *et al.,* 1999: Kabeya *et al.,* 2006). However, this mechanism is not fully understood.

1.3.4 Clinically important *Bartonella* disease

1.3.4.1 Trench fever

Trench fever is caused by infection with *B. quintana*. This bacterium is transmitted to humans by the human body louse, *Pediculus humanus* which also acts as the vector of *Rickettsia prowazekii (*Koehler *et al*., 1996). The disease occur in two forms in immunocompromised individuals and may be acute (causes trench fever) or chronic causing chronic bacteremia (Maurin *et al*., 1997). *Bartonella quintana* is common in individuals exposed to war, poverty and alcohol abuse (Billeter *et al.,* 2008). The disease is characterized by headaches, rash, dizziness and pains and repeated cycling fever associated with chronic bacteremia (Agan *et al.,* 2002).

1.3.4.2 Cat scratch disease (CSD)

Cat scratch disease is caused by an infection with *B. henselae.* This bacterium was also identified as the cause of bacillary angiomatosis (proliferation of blood vessels) and pelio hepatis (blood filled cavities in the liver or any other organ) in immunocompromised individuals, particularly HIV positive individuals (Perkocha *et al.,* 1990). The disease is characterized by swelling of the lymph nodes and fever, followed by a distal scratch or red/brown papule (lesion) (Koehler *et al*., 1997). The infection is self-limited and the swelling of regional lymph node takes 2-3 weeks (Rolain *et al*., 2004).

1.3.4.3 Carrión's disease

Bartonella bacilliformis is transmitted to humans by a sandfly, *Lutzomyia peruensis (*Amano *et al.,* 1997). *Bartonella bacilliformis* causes Carrión's disease in humans*.* The bacterium localizes in the capillary endothelial cells and in most cases primary isolations are asymptomatic (Walker *et al.,* 1999). Oroya fever occurs when the bacterium enters the erythrocytes and hemolysis occurs as a result of

erthrophagocytosis (ingestion of red blood cells) by the macrophages. Only a few patients recall acute febrile illness prior to chronic bacteremia (Rolain *et al.,* 2004).

1.3.4.4 Bacillary angiomatosis

Bacillary angiomatosis is characterized by cutaneous vascular proliferative nodules (Rolain *et al*., 2004). It was initially described in HIV-infected individuals, but it is now being reported in healthy individuals. *B. henselae* and *B. quintana* are responsible for this manifestation (Dolan *et al*., 2002). The visceral form of this disease is called pelios and may affect liver, spleen, kidneys, brain and lungs (Regnery *et al*., 1992).

1.3.5 Diagnosis

Different pathological response to *Bartonella* infections has made diagnosis difficult (Breitschwerdt *et al*., 2010). Clinical symptoms and historical information (epidemiology) are the key steps of diagnosing *Bartonella* infection. Figure 1.7 describes the guidelines used when diagnosing *Bartonella* infections

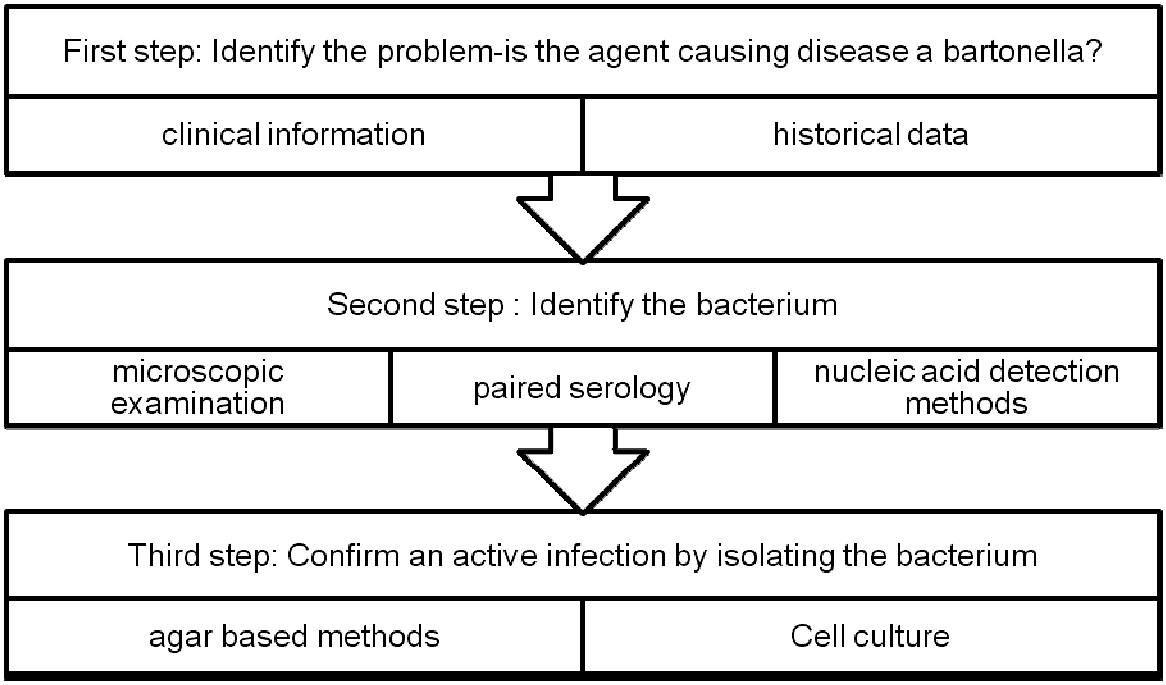


Figure 1.7: Guidelines for diagnosis of *Bartonella* infections as described by Anderson and Newmann, 1997 and Breitschwerdt *et al*., (2010)

1.3.5.1 Biosafety levels for handling *Bartonella* species

Bartonella species are highly pathogenic microorganisms and classified under the biosafety risk group 3 organisms. Risk group 3 organisms are pathogens that may present hazard to laboratory workers. They can spread in communities but there are preventative measures available. In many countries, *Bartonella* species are handled

in a Biosafety level 2 laboratories (BSL2) (Rolain *et al*., 2003). However, not all species/strains within this genus have a validated antibiotic treatment and as a result some countries use the biosafety level 3. Currently in South Africa, the National Institute for Communicable Diseases, Special Bacterial Pathogens Reference Unit (National Health Laboratory Service) is the only facility that is equipped to diagnose infections caused by these organisms in humans.

1.3.5.2 Clinical and epidemiological information

Clinical and historical information are the key factors to consider when diagnosing bartonellosis (*Bartonella* diseases) (Anderson and Neumann, 1997; Breitschwerdt *et al.,* 2010; Guptill, 2010). Clinical information involves the symptoms of *Bartonella* infection and historical or epidemiological data involves a report of arthropod bite or contact with dogs, cats or rodents. In the cases of cat scratch disease (CSD), diagnosis is based on the following criteria i) presence of swollen lymph nodes (regional lymphadenopathy), ii) history of contact with cats/cat scratches, iii) presence of histolological features and iv) a positive skin test (Anderson and Neumann, 1997; Breitschwerdt *et al.,* 2010; Guptill, 2010).

The skin test uses material obtained from lymph nodes that are heat inactivated from a patient fulfilling the diagnostic criteria of the disease. The antigenic material is injected intradermally to the patient with suspected CSD. The appearance of delayed hypersensitivity response is evaluated at 48-96 hours (Anderson and Neumann, 1997). The reaction produced by a skin test is a characteristic of hypersensitivity reaction due to cell mediated immune response. The T-cells are activated when the antigenic material is reintroduced in the body and a positive result is characterized by hardening and redness at the injection site (Resto-Ruiz *et al*., 2003).

Other *Bartonella* spp. infection (*B. henselae and B. quinatana*) results in bacillary angiomatosis (proliferation of blood vesssels) that produces cutaneous or subcutaneous lesions. Histopathological inspection of a skin biopsy can be used to confirm *Bartonella* infections. For example lobular propagation of blood vessels in the lesion can be seen by staining with haematoxylin and eosin. Other stains such as Warthin Starry silver staining have proven to be valuable for detection of bacillary angiomatosis (BA) and cat scratch disease (CSD) (Anderson and Neuwann, 1997).

In some cases diagnosing *Bartonella* infection can be difficult if the primary inoculation site has healed or is not evident and no record of animal contact is obtained. In such cases several techniques can be employed including; nucleic acid detection methods (PCR), paired serology and isolation in culture (Anderson and Neuwann, 1997).

1.3.5.3 Nucleic acid detection methods

The polymerase chain reaction can be used to directly amplify a *Bartonella* specific DNA sequence from different diagnostic samples (lymph nodes tissues, skin biopsy specimen and blood) (Matar *et al.,* 1993; Chomel and Kasten, 2010; Breitschwerdt *et al.,* 2010). In addition, *Bartonella* species can be identified by direct sequencing of the PCR product. A variety of genomic PCR targets have been described for identification and differentiation of *Bartonella*. These include the highly conserved *16S rRNA* gene (Regnery *et al.,* 1995), the protein encoding genes such as citrate synthase gene (*gltA*), riboflavin synthase gene (*ribC*), cell replication gene (*ftsZ*) and the gene encoding heat shock proteins *(groEL*) (Zeaiter *et al*., 2002; La-scola *et al.,* 2003). Polymerase chain reaction offers an advantage in the detection of *Bartonella* over blood culture and serological methods in that it is rapid, sensitive and specific (Matar *et al*., 1993; Fenollar and Raoult, 2004).

In parenchymal tissues such as spleen, kidney, liver and muscles, the sensitivity of conventional PCR is reduced because bacterial levels are potentially low. In these cases, nested and real time PCR are used to increase the sensitivity for detection of *Bartonella* DNA (Zeaiter *et al*., 2003). Nested and real time quantitative PCR for detection of *Bartonella* in clinical specimens have been developed (Zeaiter *et al*., 2003; Rampersad *et al.,* 2005). These techniques are highly sensitive and in the case of real time PCR, the DNA is detected and quantified simultaneously (Zeaiter *et al.,* 2003; Guptill, 2010).

1.3.5.4 Serology

Serology is the most common method used for labarotory diagnosis of *Bartonella* infections. Serological tests that are often used include the enzyme-linked immunosorbant assay (ELISA), Western blotting and the indirect immuno-fluorescent assay (IFA). These methods measure antibodies in body fluids (serum, plasma, or cerebrospinal fluid) (Schwatzman, 1996; Guptill, 2010; Breitschwerdt *et al*., 2010; Chomel and Kasten, 2010). Usually IgM levels become detectable within 1-2 weeks after onset of illness and rise, peaking by the fourth week and decline to

undetectable levels usually 100 days after the onset of symptoms. IgG levels become measurable shortly after IgM and peak at a higher level after eight weeks and may either persist or decline over time (Dalton *et al*., 1995; Guptill, 2010). The reciprocal titers of IgM by IFA are moderately low (32 to 128) when compared to IgG (256 to 2028) (Zbinden *et al.,* 1997; 1998; Not *et al*., 1999). In some cases individuals may have bacteremia (bacteria in the blood) with positive cultures, yet antibodies levels remain undetectable (Lucey *et al.,* 1992; Agan *et al.,* 2002). IFA and ELISA are the most commonly used tests but cannot differentiate antibody responses between different *Bartonella s*pecies. Antibody cross-absorption and Western immunoblotting may allow differentiation (Houpikian and Raoult, 2003). Because of the genetic diversity of *Bartonella* species, infections with some strains/species of *Bartonella* may not be detected using any of these methods (McCool *et al.,* 2008).

Immunofluorescent assays (IFA) are the most frequently used. Studies have confirmed that IFA detection of *B. henselae* is sensitive (approximately 95%) and specific (up to 97%) (Dalton *et al.,* 1995). The majority *of Bartonella* IgM and IgG diagnostic kits are designed to detect the antigen-antibody complexes when diluted sera are presented with *Bartonella* antigens on commercially produced slides. A fluorescein-labeled anti-IgG or IgM conjugate is used for visualization of reaction (Agan *et al.,* 2002). Although, IFA has high sensitivity for *Bartonella* antibody detection, cross-reaction with other pathogens such as *Chlamydia and Coxiella burnetti* has been reported (Raoult and Roux 1996; Maurin *et al.,* 1997; Agan *et al*., 2002; Brenner *et al.,* 2005; Vermuelen *et al.,* 2007; 2010). The other disadvantage of IFA is that it is time consuming, requires expensive equipments such as a fluorescent microscope and trained personnel are required for interpreting results (Drancourt *et al*., 1995; Vermuelen *et al.,* 2007).

Different antigen preparations (agar-derived and Vero cells cultivated) have been shown to affect the results of the IFA test (McCool *et al*., 2008; Guptill, 2010). Agar-derived antigens yield low titers when compared to cell-cultivated antigens (Agan *et al*., 2002) and auto-agglutinate when introduced in buffer saline and this may affect the sensitivity of IFA (Giladi *et al*., 2001; Agan e*t al*., 2002).

ELISA is easy to perform and use readily available equipment, which may be automated. Several publications have shown that results obtained by ELISA are fairly contradictory (Agan *et al.,* 2002). An ELISA test was developed using an agar-derived *B. henselae* (Barka *et al.,* 1993) and the sensitivity and specificity of the assay was 96% and 99%, respectively. Similar results were also reported by other studies (Patnaik and Peter, 1995). Numerous publications have shown that ELISA detection of IgG is less sensitive (Bergemans *et al*., 1997; Not *et al.,* 1999) and a higher sensitivity is obtained during ELISA detection of IgM (Bergemans *et al.,* 1997). However, recent studies have reported that the commercially available ELISA diagnostic kits for IgM detection have lower sensitivity when compared to ELISA kits for IgG detection (Vermulelen *et al*., 2007; 2010). Several studies have shown that by using the outer membrane proteins as antigens, sensitivity and specificity for IgM detection was 94% and 99%, whilst for IgG detection only 86 % and 88%, respectively (Litwin *et al.,* 1997; Agan *et al*., 2002). Results can either be visualized by naked eye or by using ELISA computerized readers. For an ELISA to be considered positive for cat-scratch disease, IgG titers should be ≥ 1:64, and for infective endocarditis the antibody titers ≥ 1:800 (Saisongkohr *et al*., 2010).

1.3.5.3 Isolation of *Bartonella*

Bartonella are highly fastidious, facultative intracellular bacteria that can be isolated by an agar-based system and in cell culture (Agan *et al.,* 2002; Bolouis *et al.,* 2005). A positive blood or other tissue culture result (lymph nodes tissues, skin biopsies) is the most reliable test for an ultimate diagnosis of active *Bartonella* infection (Guptill, 2010). *Bartonella* can be isolated effectively from blood by using standard blood agar plates (Columbia or brain heart infusion agar), 5% CO_2 rich incubation and a prolonged incubation period at 35℃ for 2-6 weeks (Br eitschwerdt *et al.,* 2010). It has been reported that isolation of *B. henselae* and *B. clarriadgeiae* from dog and human blood samples with the same approach is not sensitive because the bacteria are present in low concentration in the blood (Breitschwerdt *et al*., 2010). Subculturing from a primary isolate may even take more than 15 days before growth is achieved. *Bartonella* appears as white, adherent colonies that have different sizes and shapes on agar (Agan *et al.,* 2002).

Cell culture based methods are more sensitive and allow rapid growth of *Bartonella* when compared to agar based methods (Agan *et al.,* 2002). Cell culture methods are usually performed with a shell vial containing human endothelial cell monolayers (endothelial vascular vein-ECV 304 cells) and Vero cells (Winn, 1997). In this technique, infected tissue is homogenized in an Eagle minimal essential medium supplemented with 4% calf serum and 2mM $_{L}$-glutamine and the suspension is inoculated into monolayers (ECV 304/ Vero cells) in shell vials. The inoculated shell vials are centrifuged and incubated at 35℃ in CO_2-enriched atmosphere. Bacterial growth is detected after 15 days of incubation either by Gimenez stain or microfluorescence assay (Maurin and Raoult, 2000).

1.3.6 Treatment of *Bartonella* infections

Since clinical manifestations of *Bartonella*-induced disease are not the same, there is no single treatment for all cases. Cat scratch disease does not respond to antibiotic treatment but the infection is sometimes self limiting. Doxycycline, azithromycin and rifampin were found to be effective in patients with acute bacteremia, bacillary angiomatosis, and parenchymal peliosis (Rolain *et al*., 2004; Breitschwerdt *et al*., 2008). Other antibiotics such as penicillin, gentamycin and azithromycin are also recommended for treatment of bacillary angiomatosis and peliosis hepatitis (Rolain *et al*., 2003; 2004; Chomel and Kasten, 2010). Treatment for 2 weeks in immunocompetent individuals and 6 weeks in immunocompromised individuals are generally recommended (Chomel and Kasten, 2010). A 4-6 week course of doxycycline, erythromycin or azithromycin is generally recommended for treatment of *B. quintana* (Myers *et al*., 1984; Slater *et al.,* 1990; Rolain *et al*., 2004). Penicillin G, chroroamphenicol and erythromycin are recommended to individuals with Carrión's disease (Rolain *et al.,* 2004).

1.3.7 *Bartonella* vectors

Bartonella are transmitted to a susceptible host by a wide variety of arthropods including bitting flies, fleas, and lice (Chomel *et al.,* 2009). With regards to arthropods, there is a considerable difference between confirmed vector competence and vector potential (Billiter *et al*., 2008). Confirmed or proven vector competence is based on experimental studies which show consistent transmission between vector and the host (Billiter *et al*., 2008). In most cases detection of *Bartonella* species either by PCR or culture does not neccesarly indicate vector competence, but simply

symbolizes the ingestion of *Bartonella* infected blood from a bacteremic host (Billiter *et al.*, 2008).

1.3.7.1 Sand-flies

The sand fly, *Lutzomyia verrucarum* was proposed to be a possible vector of *Bartonella bacilliformis (*Townsend, 1914; Billiter *et al*., 2008*)*. This was based on the feeding lifestyles of *Lutzomyia verrucarum* in relation to the distribution of cases of Oroya fever in the Peruvian Andes. Following Townsend's experiments, several studies were conducted to determine if *L.verrucarum* could be a vector of *B. bacilliformis* (Battistini, 1929; Noguchi, 1929). In all these studies (Battistini, 1929; Noguchi, 1929), *B. bacilliformis*–like microorganisms were detected in the blood of the hosts that were fed upon by an infected *L. verrucarum* suggesting that transmission of *B. bacilliformis* could occur during a bloodmeal. Replication mechanisms of *B. bacilliformis* in *L. verrucarum* are not fully understood. When *L. verrucarum* feed on the infected host, *B. bacilliformis* like organisms were visible in the midgut adhering to the surface of the intestine and also in the feces. This suggests that *B. bacilliformis* replicates in the midgut of the *L. verrucarum* and transmission to other susceptible hosts could also occur via infected feces contact (Billiter *et al*., 2008).

1.3.7.2 Lice

The human body louse, *Pediculus humanus humanus* has been identified to be the potential and competent vector of *Bartonella quintana* which is an agent of trench fever. Humans are the only known vertebrate hosts of *B. quinatana* (Maurin *et al.,* 1997). The adult body louse becomes infected during a blood meal and viable microorganisms are maintained in the intestinal tract and shed in the feces. Successive spread to humans occurs through contamination of a louse bite or wound with infected louse feces (Billeter *et al.,* 2008). The head louse (*Pediculus humanis corpis)* has also been identified to be a possible vector of *B. quintana* (Sasaki *et al.,* 2006).

1.3.7.3 Fleas

Ctenocephalides felis has been experimentally confirmed to be a competent vector for transmission of *B. henselae*, which is an agent of cat scratch disease (Chomel *et al.,* 1996). These fleas are naturally infected with *B. henselae.* Bacterial replication occurs in the flea midgut and shed in the feces and can remain viable for long period. Transmission to humans occurs as a result of inoculation of *B. henselae*

contaminated feces into the skin (Billeter *et al.,* 2008). *Ctenocephalides felis* can sustain infection of *B. henselae* and spread it to other cats which can then transmit the bacterium to people through a bite or scratch (Billeter *et al.,* 2008). Other fleas such as *Ctenophthalmus nobilis* have also been reported to be potential vectors for transmission of *B. grahamii* and *B. taylorii* to other animals such as bank vole (*Myodes glareolus*) (Bown *et al.,* 2004), however the mode of transmission to these animals is not known (Chomel *et al.,* 1996). *Ctenocephalides felis* has also been reported to be a vector of *B. clarriadgeiae*, *B. quintana* and *B. koehlerae* (Koehler *et al*. 1994; Chomel *et al.,* 1996; Lappin *et al*., 2006).

1.3.8 Surveillance for *Bartonell*a infections in Africa

In Africa, there is limited information describing *Bartonella* infections in humans and animals. *Bartonella* have been detected in different hosts in Africa and the most common *Bartonella* spp. in Africa is *B. henselae.* This bacterium has been detected in domestic cats, lions, cheetahs and dogs and even humans from Gabon, South Africa and Zimbabwe (Kelly *et al*., 1998; Molia *et al*., 2004; Kelly *et al*., 2004a; Gundi *et al*., 2004; Trataris, 2011). Other *Bartonella* species that have also been reported in Africa include *B. clarriadgeiae*, *B. vinsonni subsp. berkofii, B. bovis* and *B. quintana* (La-Scola *et al.,* 2004; Gundi *et al*., 2004; Kelly *et al.,* 2005; Kerniff *et al.,* 2010; Trataris, 2011).

1.3.8.1 *Bartonella henselae*

Bartonella henselae is the causative agent of cat scratch disease (CSD). This bacterium was isolated by Dolan *et al.,* (1993) in a patient with CSD lymphadenitis (swollen lymph nodes). The serological evidence linking this bacterium as the main agent of cat scratch disease was reported by Maurin and colleagues (Maurin *et al.,* 1997). This bacterium is widespread in temperate conditions, where there is a high rate of cat flea infestations (Jameson *et al.,* 1995). It has also been identified in dogs from Gabon (Gundi *et al.,* 2004). Antibodies against *B. henselae* antigens were reported in 24% of domestic cats from South Africa and Zimbabwe (Kelly *et al.,* 1996). Subsequent studies in South Africa reported *B.henselae* in cheetahs and free-raging lions (Kelly *et al*., 1998; Molia *et al.,* 2004; Pretorius *et al*., 2004c). A recent study in Johannesburg-South Africa, reported sero-conversion against *B. henselae* antigens in HIV infected individuals, dogs and cats. In this study HIV-infected patients had 32 % IgG and 14% IgM seroprevalance and healthy individuals had 19% IgG seroprevalance. The prevalance by nucleic acid detection methods

was 22.5% in HIV infected individuals, 9.5% in healthy individuals, 23.5% in cats, 9% in dogs and 25% in rodents. *Bartonella* isolates were cultured and only cat and rodent samples yielded isolates (Trataris, 2011).

1.3.8.2 *Bartonella vinsonii subsp. berkofii*

B. vinsonii subsp. berkofii was first isolated from a female dog with endocarditis (Breitschwercht *et al*., 1996). Subsequent isolations in another dog with endocarditic was reported (Kordick *et al.,* 1997). This bacterium has been implicated in heart muscle inflammation, peliosis hepatitis (filled blood cavities in liver,) and sudden death in dogs (Kordick *et al.,* 1997). Studies in Morocco reported sero-prevalence against *B. vinsonii subsp bekoffi* in 38% of the dogs tested (Henn *et al.,* 2006). The DNA of this bacterium was recently detected in 1/80 dogs tested in Algeria (Kerniff *et al.,* 2010).

1.3.8.3 *Bartonella bovis*

Bartonella bovis has been recognized as a novel species based on phenotypic, biochemical and the *16S rRNA* and *gltA* gene diversity (Bermond *et al.,* 2002). This bacterium naturally infects cattle (Chomel and Kasten, 2010). It has also been isolated in other animals including cats (Regnery *et al.,* 2000; Breitschwerdt *et al*., 2001; Bermond *et al.,* 2002). The DNA of this bacterium was detected in zebu cattle (*Bos indicus*) in the Ivory Coast (Raoult *et al.,* 2005).

1.3.8.4 *Bartonella clarriadgeiea*

Cats are the main reservoir of this bacterium. This bacterium is also known as one of the causative agents of cat scratch disease and was initially isolated from a cat of a HIV-positive individual with cat scratch disease (Sander *et al.,* 2001). It has also been isolated from the blood of dogs with aortic endocarditis (Chomel *et al*., 2001). The DNA of this *Bartonella* species was detected in 2/80 dogs tested in Algeria (Kerniff *et al.,* 2010).

1.3.8.5 *Bartonella quintana*

Bartonella quintana (an agent of trench fever) is transmitted to humans by a body louse, *Pediculus humanus humanus* (Billeter *et al.,* 2008). It was isolated in the 1960s by Vinson (1966) from a patient in Mexico City. Sporadic reports of trench fever appeared in Europe, Asia and North Africa followed by focal outbreaks in World War II (Ohl and Spach, 2000). It has been reported to cause louse borne disease in Burundi (Raoult *et al*., 1998). A recent study in South Africa also reported antibodies

against *B. quintana* in HIV-infected individuals, cats, dogs, and rodents (Trataris, 2011).

1.3.9 *Bartonella* species in bats

Bartonella species are distributed worldwide and have been detected in wide variety of hosts including humans, felines, canines and ruminants (Kosoy, 2009). Only a few studies have reported *Bartonella* DNA in bats (Concannon *et al*., 2005; Kosoy *et al.,* 2010; Bai *et al*., 2011). The first study in Cornwall, United Kingdom reported *Bartonella* DNA in 4 different bats spp. (*Myotis mystacinus*, *Pippestillus spp*., *Myotis daubentonii* and *Nyctalus noctula*). *Bartonella* DNA was detected in 5/60 bats tested and DNA sequences generated clustered separately from previously described *Bartonella* strains (Concannon *et al.,* 2005).

In the other study in Kenya, 13 different insectivorous and frugivorous bat species were collected from different locations. A total of 331 bats were collected and *Bartonella* was cultured in 106/331 bats. From the 106 positive culture samples, 98 yielded a PCR product when subjected to a PCR targeting the partial fragment of the citrate synthase (*gltA*) gene. In total, 58 *gltA* genotypes were identified, representing 11 unique phylogroups. All the genotypes detected in bats were “species-specific”. The mode of transmission of *Bartonella* to these bats is unknown. Further investigations are needed to determine whether these agents are reponsible for human illness in that region (Kosoy *et al*., 2010).

A recent study in Guatemala, also reported *Bartonella* DNA in bats by using culturing and nucleic acid detection methods. *Bartonella* was cultured in 41/118 bats collected in this area. Twenty one *gltA* genotypes were identified and clustered into 13 phylogroups. The prevalence of *Bartonella* infection was 33% and it was assumed that this high prevalence was a result of persistent infection (Bai *et al.,* 2011). Unlike the study in Kenya (Kosoy *et al.,* 2010), *Bartonella* species detected in this study were not “species-specific”. *Bartonella* species detected in vampire bats might be responsible for undiagnosed human disease in that area, because it has been reported that these bats can feed on human blood (Turner and Bateson, 1975; Bai *et al.,* 2011).

Another recent study in Taiwan investigated the epidemiology of *Bartonella* infections in bats and other small mammals from the same ecological environment. In this study 54 bat species (*Rhinolophus monoceros*, *Hipposideros terseness, Pipistrellus abramus* and *Miniopterus schreibersii*) were collected in Taiwan. *Bartonella* was only isolated from four *Miniopterus schreibersii* blood samples. Sequence and phylogentic analysis of the *16S rRNA, gltA, ribC, rpoB* and *ftsZ* genes indicated that the genotypes obtained were not similar to *Bartonella* genotypes detected from *M. schreibersii* in Kenya, but was related to *Bartonella* spp. from dogs in Taiwan. Further investigations are needed to determine whether *Bartonella* could be transmitted between bats and dogs (Lin *et al.,* 2011).

1.3.10 *Bartonella* and bat ectoparasites

Limited publications have reported *Bartonella* in bat ectoparasites. Loftis *et al.,* (2005), reported *Bartonella* DNA in a bat tick *Carios kelleyi* in Jackson County, Iowa (USA). In another study, Reeves *et al.,* (2005) reported *Bartonella* DNA in *Streblidae* and *Cimidae* (bat flies). In this study, *Bartonella* DNA was detected in one *Trinchobius major* (*Diptera: Streblidae*) collected from a *Myotis austropiparius* bat from Florida Caverns State parks and one *Cimex adjunctus* (*Hemiptera; Cimidae*) collected from Sante caves, Orangeburg County (USA) in a *Myotis austropiparius* roost. These ectoparasites have been reported to bite humans when they enter the bat roosts (Lloyd, 2002). A recent study in Ghana and Gulf of Guinea reported the detection of *Bartonella* DNA in bat flies *Cyclopodia greefi greefi (Diptera: Nycteribiidae)* that were removed from *Eidolon helvum* bats. In this study, 66.4% (91/137) bat flies tested positive for *Bartonella* by PCR. In addition, viability of the bacterium was demonstrated in one bat fly (Billeter *et al.,* 2012).

1. 4 Aims and objectives of the study

1.4.1 Aim

The aim of the study was twofold:

- Evaluation and comparison of PCR assays for detection of *Rickettsia* and *Bartonella.*
- To detect *Rickettsia* and *Bartonella* from a panel of blood specimens collected from different bat species in South Africa and Swaziland using PCR.

1.4.2 Objectives

- Evaluation of PCR assays necessary for detection of *Rickettsia* and *Bartonella.*
- Optimization of PCR assays (targeting the *gltA* and *16S rRNA* genes) for detection of *Bartonella* and *Rickettsia* DNA.
- Development of synthetic controls for the assays.
- To detect *Rickettsia* and *Bartonella* from a panel of blood specimens collected from different bat species in South Africa and Swaziland using PCR.
- Partial molecular characterization and analysis of phylogeny of any *Rickettsia* and *Bartonella* species detected and comparison with those from previous studies reported in the literature.

Chapter 2: Evaluation of *Rickettsia* and *Bartonella* nucleic acid detection methods.

2.1 Introduction

Bartonella and *Rickettsia* are arthropod-borne bacteria that are associated with mammalian hosts (Fournier and Raoult, 2009; Guptill, 2010). At least 13 species within these two genera are known to cause human diseases worldwide (Parola *et al.,* 2005; Breitschwerdt *et al.,* 2010). These organisms are associated with a growing spectrum of emerging and re-ermerging diseases in Africa (Frean *et al.,* 2008; Trataris, 2011). The most common bartenellosis and rickettsioses in South Africa are tick bite fever and cat scratch disease, respectively (Frean *et al.,* 2002; 2008).

Laboratory diagnosis of *Bartonella* and *Rickettsia* infections relies on the serological tests (IFA and EIA), culture techniques and/or molecular tools (Agan *et al.,* 2002; Parola *et al*., 2005). Serology is the most practised diagnostic tool for *Rickettsia* and *Bartonella* in many developing countries, including South Africa. The drawback of serological testing is that it only allows identification of *Rickettsia* and *Bartonella* to the group or clade level and often results are delayed because antibody levels are only detectable when the patient is recovering or in the acute stages of infection (La Scola and Raoult; 1997; Agan *et al*., 2002; Parola *et al*., 2005; Frean *et al.,* 2008; Kowalczeska *et al.,* 2012). *Rickettsia* and *Bartonella* are fastidious and therefore, isolation in culture is difficult and requires lengthy incubation periods (Agan *et al.,* 2002; Parola *et al.,* 2005; Wölfel *et al.,* 2008; Guptill, 2010). Most of rickettsial and bartonella infections are only diagnosed clinically, but few are laboratory confirmed. PCR could be used in conjunction with serology to have a confirmed diagnosis. Molecular tools (PCRs) offer advantages because they circumvert the need to culture and offer a higher sensitivity and specificity as well as direct detection of bacterial DNA in clinical samples (Agan *et al*., 2002; Watt and Parola, 2003; Jiang *et al*., 2004; Stenos *et al.,* 2005; Paris *et al.,* 2007; Guptill, 2010). Additional benefits of PCR testing are that species or strain of *Bartonella* and *Rickettsia* may be identified by sequencing of the amplified product (Fenollar and Raoult, 2004).

A number of genomic PCR targets have been described for identification and differentiation of *Rickettsia* and *Bartonella* (Agan *et al.,* 2002; Fournier and Raoult, 2009). Among all the genes identified and characterized, the *16S rRNA* and the *gltA* gene are the most commonly used genes for identification of *Rickettsia*. The *16S rRNA* gene is the housekeeping gene that evolves at a slow rate and is useful for identification of *Rickettsia* to the genus and the group level (Roux and Rauolt, 1995; Fournier *et al.,* 2003, Fournier and Raoult, 2009). The level of sequence similarity among *Rickettsia* species are high (>98%) and therefore, studies of this gene does not provide accurate identification to the species level (Fournier and Raoult, 2009). This gene is usually used to confirm results obtained from protein encoding genes (Roux and Raoult, 1995). The citrate synthase gene *(gltA*) gene is conserved among *Rickettsia* species and has been previously used to identify closely related *Rickettsia* species (Fournier and Rauolt, 2009).

Identification of *Bartonella* species relies on the analyses of the *gltA, ftsZ*, *ribC*, *groEL*, *16S rRNA* genes and the *16S/23S* intergenic spacers (Agan *et al.,* 2002; Zeaiter *et al*., 2002; Johnson *et al*., 2003). PCR based on the the *16S rRNA* and the *16S/23S* intergenic spacers are frequently intricated by intraspecies heterogeneity observed in these regions. The levels of sequence similarity are high (>98%) and therefore provide accurate identification of *Bartonella* to the genus level. Phylogenetic studies of *Bartonella* based *groEL*, *FtsZ,* and *ribC* genes has also been demonstrated (Zeaiter *et al*., 2002; Johnson *et al*., 2003) and among the genes described, the *gltA* has been reported to be the best tool for identification of *Bartonella* (Paziewska *et al*., 2010). The gene sequences of this enzyme are conserved in the genus *Bartonella* and have been previously used to estimate divergence among closely related *Bartonella* species (Birtles and Raoult, 1997). This gene was recently used to detect *Bartonella* DNA in bats for the first time in Kenya, Guatemala and Taiwan (Kosoy *et al*., 2010; Bai *et al*., 2011; Lin *et al.,* 2011). All these genes mentioned have been successfully used to identify *Bartonella* and *Rickettsia* species in Africa, including South Africa (Pretorius *et al.,* 2004a; Pretorius *et al*., 2004c; Frean *et al.,* 2008).

In some cases (early or recovery stages of infection) when circulating bacterial pathogens are potentially low, the sensitivity of conventional PCR is reduced (Wölfel *et al.*, 2008; Guptill, 2010). In these cases nested and real time PCR increases sensitivity. Nested and real time PCR assays for *Bartonella* and *Rickettsia* have

been described (Zeaiter *et al.,* 2003; Jiang *et al.,* 2004; Rampersad *et al.,* 2005; Kidd *et al.,* 2008; Wölfel *et al*., 2008). These techniques are not only sensitive but they are also fast and in cases of a real time PCR, bacterial DNA is detected and quantified simultaneously (Wölfel *et al*., 2008).

The aim of the chapter was to evaluate and improve previously reported PCR assays necessary for the detection of *Rickettsia* and *Bartonella* DNA (Perotti *et al*., 2006; Wölfel *et al.,* 2008; Kosoy *et al*., 2010). These assays will be useful in future as surveillance tools and will be further implemented in chapter 3 and 4 for the detection of these pathogens in a panel of bat blood samples.

2.2 Materials and Methods

2.2.1 Evaluation of PCR assays for detection of *Rickettsia*

2.2.1.1 *Rickettsia* strains

The extracted genomic DNA of *Rickettsia conorii* was provided by Dr Jacqueline Weyer of the Centre for Zoonotic and Emerging diseases (Previously Special Pathogens Unit) National Institute for Communicable Diseases (National Health Laboratory Service) Sandringham, Johannesburg, South Africa.

2.2.1.2 Primers

Primers used in this section are indicated in Table 2.1. The primer sequences used for detection of the *16S rRNA* gene of *Rickettsia* were obtained from Perotti *et al.,* (2006). The sequences of primers used for detection *gltA gene* of *Rickettsia* in conventional and real time PCR were obtained from Wölfel *et al.,* (2008) and the sequences of primers used for detection *gltA* gene of *Rickettsia* in nested PCR were designed by Dr Jacqueline Weyer of the Centre for Zoonotic and Emerging diseases (previously Special Pathogens Unit) National Institute for Communicable Diseases (National Health Laboratory Service). The PCR conditions were designed based on the PCR product length and the melting temperature of the primers (Table 2.1)

Table 2.1: Oligonucleotide primers used for PCR amplification and DNA sequencing of the *gltA* and *16S rRNA* gene of *Rickettsia* species

Primer name	Primer sequences (5'-3' direction)	Position on the genome in bp	Melting temperature in℃	Target gene	Type of PCR	PCR conditions
* NgltF (use with NgltR)	GTATATTCCTAAATATATAGC	884-910 *R. conorii* strain Malish 7 (NC 003101.1)	43.7	Citrate synthase (*gltA*)	Nested (Amplification and sequencing)	94℃ for 2 min, followed by 40 cycles of 94℃ for 30s, 40℃ for 30sec, 72℃ for 30s and then 72℃ for 5 min
# NgltR (use with NgltF)	GTTCTATTGCTATTTGTAAG		40.1			
* 16SF (use with 16SR) (Perotti *et al*., 2006)	ASGCGGTCATCTGGGCTACAACT	659-1059 *R. conorii* strain Malish 7 (L36107)	57.9	16SrRNA	Conventional (Amplification and sequencing	94℃ for 5 min, followed by 35 cycles of 94℃ for 1 min , 55℃ for 1min sec, 72℃ for 1.5 min and then 72℃ for 10 min
#16SR (use with 16SR)	CCGCTGGCAAATAAGAATGAGG		60.2			
* PanRick2 (use with PanRick2b and PanRick 7	ATAGGACAAACCGTTTATTT	4332-4352 *R.conorii* strain Malish7 (AE008677)	46.1	Citrate synthase (*gltA*)	Real time	95℃ for 10in followed by 45 cycles of 95℃ for 10 sec, 40℃ for 30 sec 72℃ for 3 sec and then 40℃ for 30 sec
# PanRick 2b (use with PanRick2 and PanRick 7)	CAACATCATATGCAGAAA	4383-4401 *R.conorii* strain Malish7 (AE008677)	44.7			
$ PanRick 7 use with PanRick2 and PanRick 2b) (Wölfel *et al.,* 2008)	FAM-CCTGATAATTCGTTAGATTTTACCG-BHQ	4332-4401 *R.conorii* strain Malish7 (NC 003101.1)	51.8			
* PanRick2 (use with RpCs 1258)	CAACATCATATGCAGAAA	4383-4401 *R.conorii* strain Malish7 (AE008677)	46.1	Citrate synthase (*gltA*)	Conventional (Amplification and sequencing)	94℃ for 5min, followed by 30 cycles of 94℃ for 30s , 50℃ for 30 sec and 72℃ for 30 sec and then 72℃ for 5 min
# RpCs1258 (use with PanRick2)	ATTGCAAAAAGTACAGTGAACA	1137-1258 *R.conorii* strain Malish7 (AE008677)	51.1			

*-indicates forward primer, # indicates reverse primer, $ indicates a probe

2.2.1.3 Evaluation of primer sequences

To evaluate whether these primer sequences listed in Table 2.1 could be used to amplify the species or strains of *Rickettsia* from Africa, representative sequences were obtained from GenBank (NCBI) (Table 2.2 and 2.3). ClustalW was used to create sequence alignments (Hall, 1999). Sequences of *Rickettsia* (*16S rRNA*) from other countries were also included, because not the all rickettsial species reported in Africa have *16S rRNA* sequences available in the public domain.

Table 2.2: The *gltA* gene sequences of African *Rickettsia* species used to evaluate primer sequences derived from previous studies

***Rickettsia* species**	**Strain**	**Host**	**Country**	**Genbank acession number**	**Reference**
R. sibirica mongolotimonae	URRMTMMFE	*Homo sapiens* (Human)	Algeria	DQ097081	Fournier and Raoult, 2007
R.aeschlamanii	RH5	*Rhipicephalus evertsi evertsi* (ticks)	Senegal	HM582437	Mediannikov *et al.,* 2010
R. africae	ESF-5	*Amblyomma variegatum* (ticks)	Ethiopia	U59733	Roux *et al*., 1996
R. conorii	Seven	*Homo sapiens* (Human)	South Africa	U59730	Roux *et al*., 1996
R. felis	Et90	*Homo sapiens* (Human)	Ethiopia	JN366415	Unpublished
R. prowazekii	Cairo 3	*Homo sapiens* (Human)	Egypt	DQ9626853	Ge *et al*., 2004
R. felis	Pan	Not specified	Panama	HM582437	Bermudez *et al.,* 2011

Table 2.3: The *16S rRNA* gene sequences of *Rickettsia* used to evaluate primer sequences derived from previous studies.

***Rickettsia* species**	**Strain**	**Host**	**Genbank acession number**	**Country**	**Reference**
R. conorii	Seven	*Homo sapiens* (Human)	AF541999	South Africa	Roux and Raoult, 1995
R.conorii	Morrocan	*Homo sapiens* (Human)	L36105	Morocco	Roux and Raoult, 1995
R. africae	ESF-5	*Amblyomma variegatum (ticks)*	L36098	Ethiopia	Raoult and Roux, 1995
R. sibrica	246	*Dermacentor nutallii* (ticks)	L36218	USA	Raoult and Roux, 1995
R. prowazekii	Breinl	*Dermacentor variabilis* (ticks)	NR0446856	USA	Weinsburg *et al.*, 1989
R .felis	Asn1	*Liposcelis bostrychophila* (booklouse)	GQ329878	Australia	Behar *et al.,* 2010

2.2.1.4 Optimization of the PCR assays for detection of *Rickettsia*

PCR assays for detection of *Rickettsia* were initially optimized using different parameters following a modified Taguchi method (Cobb and Clarkson, 1994). Optimization focused on the $MgCl_2$, primer, template and dNTP concentrations (Table 2.4) as well as the cycling conditions using the genomic DNA of *Rickettsia conorii.*

Table 2.4: Parameters used for optimization of the PCR assay for detection of *Ricketttsia*

Parameter	Concentrations							
Template concentration (ng/µl)	1	2	5	10	15	20	25	30
$MgCl_2$ concentration (mM)	3	4	5	6	7	8	9	10
Primer concentration (pmol)	5	10	15	20	25	30	-	-
Anealling temperature (℃)	37	40	42	45	47	50	55	65
Probe concentration (pmol) (real time PCR only)	2	4	6	8	10	-	-	-
dNTP (mM)	2	4	6	8	10	20	-	-

-: indicates not tested beyond the previous concentration

2.2.1.5 Real time PCR targeting a partial *gltA* gene of *Rickettsia conorii*

This assay was carried out using the Lightcycler TaqMan Master Kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. Five µl (1ng/µl) of *R. conorii* genomic DNA was added to the master mix comprising of 4 µl of TaqMan master-mix [FastStart *Taq* polymerase, reaction buffer, $MgCl_2$ and dNTPs mix (with dUTP instead of dTTP)], 1 µl of reverse and forward primers (10 pmol PanRick 2, PanRick 2b) (Table 2.1) (IDT), 0.4 µl of oligonucleotide probe (10 pmol, PanRick7) (Table 2.1) (IDT) and 8.6 µl of PCR grade nuclease free water (Roche Diagnostics, Germany) to make a final volume of 20 µl. The oligonucleotide probe was labelled with a flourescine amidite dye (FAM) and Black hole quencher (BHQ) that has a fluorescent wavelength of 520nm. Amplification was carried in out in the LightCycler® 1.5 thermocycler (Roche Diagnostics, Germany) under the following conditions: Pre-incubation at 95˚C for 10 minutes, followed by 45 cycles of denaturation at 95˚C for 10 seconds, annealing at 40˚C for 30 seconds, extension at 72˚C for 3 seconds and final cooling at 40˚C for 30 seconds. Negative control was included in this assay and it contained all the components of the mastermix, except that the template was substituted by nuclease free water (Promega, USA). The second derivative maximum

method of the LightCycler® software version 4.05 was used for analysis of fluorescence.

2.2.1.6 Conventional PCR targeting the partial *gltA* gene of *Rickettsia conorii*

Two µl (10ng/µl) of *R. conorii* genomic DNA was added into the master mix comprising of 5µl 10 X Dream *Taq* polymerase buffer (100mM KCl, 100mM $(NH4)_2$ SO_4 and 20 mM $MgCl_2$) (Fermentas, USA), 5 µl of 2mM dNTP mix (Fermentas, USA), 1 µl of forward primer (10 pmol, PanRick2) (Table 2.1) (IDT), 1 µl of reverse primer (RpCs1258, 10 pmol) (Table 2.1) (IDT), 0.3 µl Dream *Taq* polymerase (1.25 U/µl) (Fermentas, USA) and 32.5 µl nuclease free water (Promega, USA) to make a final volume of 50 µl. A negative control with all the components of the mastermix except that the template was substituted by nuclease free water (Promega, USA) was included. Amplification was carried out in a DNA Thermo cycler (ABI-gene Amp-2720) (Applied Biosystems, Germany) under the following conditions: Initial denaturation at 94℃ for 5 minutes followed by 30 cycles of denaturation at 94℃ for 30 seconds, annealing at 48℃ for 30 seconds, extension for 72 ℃ for 1 minute and final extension at 72℃ for 7 minutes.

2.2.1.7 Agarose gel electrophoresis

Once amplification was complete, 5 µl of the PCR products were analyzed on a 2% agarose gel stained with ethidium bromide (10µg/ml) (Sigma-Aldrich, USA). The gel was resolved at 100V in an ENURO^{TM} horizontal gel box (Labnet International, USA). A DNA molecular weight marker, 100 bp Plus DNA ladder (Fermentas, USA) was used to determine the size of the products and visualized under a UV light.

2.2.1.8 Nested PCR targeting the partial *gltA* gene of *Rickettsia conorii*

The nested PCR was performed using a similar method as described for the conventional PCR with a few modifications. One µl of the PCR product (from section 2.2.1.6) was added to a mastermix described in section 2.2.1.6, except that the primers used were NgltF and NgltR (10 pmol each) (Table 2.1) (IDT). Negative control was included in this assay and it contained all the components of the mastermix and the template was substituted by nuclease free water (Promega, USA). The reaction was carried out in a DNA Thermo cycler (ABI-gene Amp-2720) (Applied Biosystems, Germany) under the following conditions: Initial denaturation at 94℃ for 2 minutes

followed by 40 cycles of denaturation at 94℃ for 30 seconds, annealing at 42˚C for 30 seconds, extensions at 72℃ for 30 seconds and final extension at 72˚C for 5 minutes. Once amplification was complete, the products were analyzed by agarose gel electrophoresis as described in 2.2.1.7.

2.2.1.9 Conventional PCR targeting the partial *16S rRNA* gene of *Rickettsia conorii*

Two µl (10ng/µl) of *R. conorii* genomic DNA was added into the master mix comprising of 0.3 µl of Dream *Taq* polymerase (1.25 U/µl) (Fermentas, USA), 5 µl of 10 X Dream *Taq* polymerase buffer (100mM KCl, 100mM $(NH_4)_2$ SO_4, 20mM $MgCl_2$) (Fermentas, USA), 1 µl of both reverse and forward primers (16SF and 16SR;10 pmol each) (Table 2.1) (IDT) and 32.5 µl of nuclease free water (Promega, USA) to make a final volume of 50 µl. Amplification was carried out in a DNA Thermo cycler (ABI-gene Amp-2720) (Applied Biosystems, Germany) under the following conditions: Initial denaturation at 95℃ for 5 minutes followed by 35 cycles of denaturation at 95℃ for 1 minute, annealing at 55˚C ℃ for 1 minute, extension at 72 ° C for 2 minutes and final extension at 72℃ for 10 minutes. Once amplification was complete , the products were analyzed by agarose gel electrophoresis as described in 2.2.1.7.

2.2.1.10 Purification of the PCR products

The PCR products were purified using the Wizard SV gel and PCR clean up system (Promega, USA) according to the manufacturer‘s instructions. Briefly, the PCR product was excised from the agarose gel and transferred to a microcentrifuge tube. Membrane binding solution (4.5M $C_2H_6N_4S$, 0.5M CHCOOK) was added at a ratio of 10 µl of membrane binding solution per 10mg of gel slice. The tubes were vortexed and incubated at 59℃ until the gel has completely dissolved. The dissolved gel mixture was transferred to a SV minicolumn and incubated at room temperature for 1 minute. The SV minicolumn was centrifuged at 13 400 x *g* for 1 minute and the flow through was discarded. The column was washed with 700 µl membrane wash solution (10 mM CH_3COO K, 16.7 µM EDTA (pH 8), 95% ethanol) and centrifuged for 1 minute at 13 400 x *g*. The wash was repeated with 500 µl of membrane wash solution and then the mixture was centrifuged for 5 minutes at 13 400 x *g*. The columns were centrifuged again for 1 minute with the microcentrifuge lid open to evaporate residual ethanol. DNA was eluted from the column with 25 µl of nuclease free water (Promega, USA) after incubation for 1 minute and centrifugation for 1 minute at 13 400 x g. To

analyze if the purification was successful, the products were analyzed by agrose gel electrophoresis as described in 2.2.1.7.

2.2.1.11 DNA sequencing of the PCR product

Automated sequencing was performed using the ABI Prism BigDye® Terminator Version 3.1 cycle sequencing kit (Applied Biosystems, Germany). The concentrations of purified amplicons were initially determined with the NanoDrop™ 1000 spectrophotometer (Fermentas, USA). The amplicons were sequenced in both directions using these primers listed in Table 2.1, PanRick 2 and RpCs1258 (for conventional PCR targeting a partial *gltA* gene *of R.conorii),* NgltF and NgltR (for nested PCR targeting a partial *gltA* gene of *R. conorii* gene) and 16SF and 16SR (for conventional PCR targerting a partial *16S rRNA* gene of *R. conorii*). Briefly 1 µl (100ng/µl) of the PCR product was added to 2 µl of Terminator reaction pre-mix (2.5X) (Applied Biosystems, Germany), 1 µl of sequencing buffer (Applied Biosystems, Germany), 1 µl of forward/reverse primer (3.2 pmol final concentration, Table 2.1) and 5 µl of nuclease free water (Promega, USA) to make a final volume of 10 µl. The reactions were placed in a DNA Thermo cycler (ABI-geneAmp-2720) (Applied Biosystems, Germany) and reactions were run under the following conditions: Initial denaturation at 94 ℃ for 1 minute, followed by 25 cycles of denaturation at 94℃ for 10 seconds, annealing at 50℃ for 5 seconds and elonga tion at 60℃ for 4 minutes and final holding at 4℃. The products were purified using the EDTA/NAOAc/ETOH method according to the BigDye Terminator v3.1 cycle sequencing protocol (Applied Biosystems, Germany). Briefly, 1 µl of 125mM EDTA, 1 µl of 3M sodium acetate and 25 µl of 100% non-denatured ethanol were added sequentially into each reaction. The mixtures were briefly vortexed and incubated at room temperature for 15 minutes. The samples were centrifuged at 13 400 x *g* at 4℃ for 30 minutes and the supernatant was carefully discarded. The pellet was washed with 100 µl of 70% ethanol and centrifuged at 13 400 x *g* for 15 minutes. The pellet was air-dried for 20 minutes. The samples were submitted to the Natural and Agricultural Sequencing Facility, University of Pretoria, South Africa, where they were analyzed on an ABI 3100 DNA automated capillary sequencer (Applied Biosystems, Germany).

2.2.1.12 Phylogenetic analysis

The sequences were edited and trimmed using the CLC Main Workbench 6 software (CLC bio A/S, Denmark). Pairwise alignment was also performed using the CLC Main Workbench 6 (CLC bio A/S, Denmark), to determine similarity between the *gltA* and the *16S rRNA* gene sequence of *R. conorri* obtained in this study and other sequences of *R. conorii* available in the public domain (GenBank; NCBI).

2.2.2 Generation of positive controls for the partial *gltA* and *16S rRNA* gene of *Rickettsia*

2.2.2.1 Ligation of the partial *gltA* and *16S rRNA* genes of *R. conorii* into a pGEM T Easy vector

The partial *gltA* and the *16S rRNA* gene PCR products of *Rickettsia conorii* were cloned into a vector using the pGEM-T-Easy kit (Promega, USA), according to the manufacturer's instructions. The ligation reactions were divided into an experimental, positive and background control. All the ligation reactions contained the following: Five µl of 2 X Rapid ligation (60mM Tris-HCl (pH 7.8 at 25℃), 20 mM $MgCl_2$, 20 mM DTT and 2 mM ATP,10% Polyethylene glycol), 1 µl of pGEM–T Easy vector (50 ng/µl) and 1 µl T4 DNA Ligase (3 U/µl). Two µl of control PCR product derived from *Pseudomonas aeruginosa* (4 ng/µl) was added in the positive control tube and 1 µl (25 ng/µl) of PCR product (from section 2.2.1.6- 2.2.1.9) was added in the experimental reaction. The template was substituted by nuclease free water (Promega, USA) in the background control. Nuclease free water (Promega, USA) was added to make the final volume in the reaction mixtures to be 10 µl. The reaction mixtures were incubated at room temperature for 1 hour and subsequently incubated at 4℃ overnight to obtain maximum ligation.

2.2.2.2 Transformation of *E. coli* JM109 high efficiency competent cells

The ligation reactions were transformed into *E. coli* JM 109 efficiency competent cells (Promega, USA) according to the manufacturer's instructions. The ligation reactions were briefly centrifuged and 2 µl of each ligation reaction was transferred to sterile 1.5 µl microcentrifuge tubes on ice. Fifty microliters of competent cells *E. coli* JM109 high efficiency (Promega, USA) was transferred to each tube and were then gently flicked and incubated on ice for 20 minutes. The cells were heat-shocked for 50 seconds in a 42℃ heating bath and placed on ice for 2 minutes. SOC medium (composition as per

manufacturer's instructions) was added and incubated for 1.5 hours at 37℃ with agitation at 150 rpm. Hundred microlites of each transformation culture, 10 µl of 100mM IPTG (Sigma-Aldrich, SA) and 40 µl of X-Gal (20 mg/µl) (Promega, USA) were plated on duplicate Luria Bertani/Ampicillin (100 ng/µl) plates and incubated at 37℃ overnight. The recombinants appeared as white colo nies, and non-recombinants appeared as blue colonies. The white colonies were selected and were grown in Luria Bertani/Ampicilin (100 ng/µl) broth at 37 ℃ in a sha ker incubator overnight in order to isolate the plasmid DNA.

2.2.2.3 Isolation of plasmid DNA

The plasmid DNA was isolated using the Gene-Jet plasmid miniprep kit (Fermentas, USA) according to the manufacturer's instructions. The bacterial culture grown overnight was centrifuged for 5 minutes at 13 400 x *g* to collect the pellet. The pellet was dissolved in 250 µl of resuspension solution (with RNase A). Bacterial cells were lysed with 200 µl lysis solution. Three hundred microliters of neutralization solution was added and immediately mixed by inverting the tube 4-6 times. The mixture was centrifuged for 5 minutes at 13 400 x *g* and the supernatant was transferred to the Gene-Jet spin column. The supernatant was further centrifuged for 1 minute at 13 400 x g and the flow-through was discarded. The column was washed twice with 500 µl of wash solution (with 75% ethanol) and centrifuged at 13 400 x *g* for 60 seconds. The column was centrifuged again at 13 400 x *g* for 1 minute to remove residual ethanol. Plasmid DNA was eluted with 50 µl of elution buffer (10 mM Tris-HCl, pH 8.5), followed by incubation for 2 minutes and centrifugation at 13 400 x *g* for 2 minutes. To analyze if isolation was successful, agarose gel electrophoresis was performed as described in section 2.2.1.7.

2.2.2.4 DNA sequencing of the plasmid

The plasmid DNA was sequenced using 10 pmol of the M13 forward primer (-21) (Applied Biosystems; Germany) as described in section 2.2.1.11, in order to ensure that PCR products were successfully cloned and to verify the orientation of the cloned product. The sequences were edited and trimmed using CLC Main Workbench 6 software (CLC bio A/S, Denmark). Pairwise alignment was performed by using the using the Bioedit v 7.1.3 software (Hall, 1999) to determine similarity between *gltA* and the *16S rRNA* gene sequence in the plasmid and the sequences obtained from section 2.2.1.12.

2.2.3 Detection limit of the PCR assays for detection of *Rickettsia*

The detection limit of the PCR assays for detection of *Rickettsia* was determined by using a modified method of creating standard curves with plasmid DNA templates for use in quantitative PCR (Applied Biosystems; Germany). The concentration of the recombinant plasmid DNA of the partial *gltA* (for real time PCR, nested and conventional PCR) and the partial *16S rRNA* gene of *Rickettsia* was determined with the NanoDrop^{TM} 1000 spectrophotometer (Thermo Scientific, USA) and was 217.5 ng/µl and 198.5 ngµl, respectively. This corresponded to 4.12e-8 copies/µl (*gltA* products) and 3.75e-8 copies/µl (400 bp *16S rRNA* product). The following steps were followed to determine the detection limit of each PCR assay.

The mass of a single plasmid molecule was initially determined as follows:

m= [n] [1.096e-21g/bp] where m=mass, n= plasmid size + insert and e-21=$\times 10^{-21}$

The mass of plasmid containing the copy number of interest was calculated as follows:

Copy number x mass of the plasmid = mass of plasmid needed

The concentration needed to achieve the required masses of the DNA was calculated as follows:

$$\textbf{Final concentration of the plasmid DNA needed} = \frac{\textbf{mass of plasmid needed (g)}}{\textbf{volume added per reaction (µl)}}$$

The serial dilutions were calculated as follows using the formula

$$C_1V_1=C_2V_2$$

Table 2.5 summarizes the volumes and concentrations of the plasmid DNA required to achieve appropriate copy number of the partial *gltA* gene of *Rickettsia* used for testing.

Table 2.5: The volumes and concentrations of recombinant plasmid DNA for partial *gltA and 16S rRNA* of *Rickettsia* needed to achieve appropriate copy numbers of the target genes in real time PCR.

Dilution number	Source of plasmid DNA for dilution	Initial concentration (g/µl)	Volume of plasmid DNA (µl)	Volume of diluent (µl)	Final volume (µl)	Final concentration (g/µl)	Resulting copy of *gltA/16S rRNA* gene sequence
0	Stock	217.5e-9	10	90	100	217.5e-10	NA
1	10^{-1}	217.5e-10	37.8	62.2	100	8.2e-9	10^{10}
2	10^{-2}	8.2e-9	10	90	100	8.2e-10	10^{9}
3	10^{-3}	8.2e-10	10	90	100	8.2e-11	10^{8}
4	10^{-4}	8.2e-11	10	90	100	8.2e-12	10^{7}
5	10^{-5}	8.2e-12	10	90	100	8.2e-13	1000000
6	10^{-6}	8.2e-13	10	90	100	8.2e-14	100000
7	10^{-7}	8.2e-14	10	90	100	8.2e-15	10000
8	10^{-8}	8.2e-15	10	90	100	8.2e-16	1000
9	10^{-9}	8.2e-16	10	90	100	8.2e-17	100
10	10^{-10}	8.2e-17	10	90	100	8.2e-18	10
11	10^{-11}	8.2e-18	10	90	100	8.2e-19	1

The reactions were performed in triplicate and a no-template negative control was included in the analysis. A similar procedure was followed for the conventional and nested PCR targeting the partial *gltA* gene and *16S rRNA* of *Rickettsia*. The cycling conditions were described in section 2.2.1.5-2.2.1.9.

2.2.3.1 Construction of standard curve for real-time PCR

The recombinant plasmid DNA for the partial *gltA* gene was serially diluted in nuclease free water (Promega, USA) representing 1-10^{10} copies/µl. Each dilution was tested in triplicate in a single run using the TaqMan Master Kit (Roche Diagnostics, Germany) (Section 2.2.1.5). The standard curve was constructed by plotting the Cp values versus the log concentration of the target using the LightcyCycler software version 4.05 (Roche Diagnostics, Germany).

2.2.3.2 Real time PCR statistical analysis

The performance of the real time assay was analyzed statistically with regards to PCR efficiency, limit of detection, precision, standard deviation and coefficient variation (Bustin *et al*., 2009).

PCR efficiency: Rate at which an amplicon is generated and it is determined by using the slope of the log linear portion of the standard curve. It is determined as follows:

$$\text{PCR efficiency} = 10^{1-/\text{slope}} - 1.$$

Ideally the efficiency of the PCR assay should be 90-100% meaning doubling of the amplicon at each cycle and this corresponds to a slope of -3.1 to -3.6 in the Ct vs log template amount standard curve (Bustin *et al*., 2009).

Standard deviation (SD): Used to perform statistical tests on experimental results and are often required to evaluate whether the observed fold differences in nucleic acid are statistically significant or simply the variation inherent in the assay.

Limit of detection (LOD): The lowest concentration at which 95% of the positive samples are detected. This means that within a group of triplicates containing the target at the concentration of LOD, no more than 5% failed reactions should occur (Bustin *et al.,* 2009).

Precision: Degree to which the repetitions of the PCR assay produce similar quantitative values. It is influenced by several factors including the temperature difference affecting completion of anealling temperature and concentration difference introduced by errors in pippeting and etc. This is usually determined by the standard deviation (SD) and the assay is considered to be precise if the standard deviation is low (<0.3) (Bustin *et al.,* 2009).

Coefficient variation (CV): Measurement of experimental variation. Intra-assay CV quantifies the amount of errors seen with the same assay (in triplicates) and inter-assay CV quantifies the errors between separate assays. It is the standard deviation expressed as a percentage of the mean (Bustin *et al.,* 2009).

2.2.4 Development of PCR assay for detection of *Bartonella*

In this section the PCR assay was only developed for the *gltA* gene, because this gene has been previously used to identify *Bartonella* from the genus to the species level.

2.2.4.1 *Bartonella* strains

The genomic DNA of *Bartonella vinsonii*, *Bartonella henselae*, *Bartonella elizabethae* and *Bartonella grahamii* were provided by Dr Jenny Rossouw of the Special Bacterial Pathogens Reference Unit, National Institute for Communicable Diseases (National Health Laboratory Service) Sandringham, South Africa.

2.2.4.2 Primers

Primer sequences used are indicated in Table 2.6. The sequences of primers used for detection of the *gltA* gene of *Bartonella* were obtained from Kosoy *et al.,* (2010). The PCR conditions were designed according to the length of the PCR product and the melting temparature of the primers and these are listed in the Table 2.6.

Table 2.6: The oligonucleotide primers used for PCR amplification and DNA sequencing of the partial *gltA* gene of *Bartonella* species

Primer name	Sequence in 5'-3'	Target gene	PCR conditions	Melting tamparature	Reference
#BhCS781.p	GGGGACCAGCTCATGGTGG	Citrate synthase (*gltA*)	94℃ for 5 min, followed by 30 cycles of 94℃ for 30s, 50℃ for 30s, 72℃ for 30 s and then 72 for 7 min	60.8	Kosoy *et al*., 2010
*BhCs1137.n	AATGCAAAAAGAACAGTAAACA			49.3	

# indicates forward primer * indicates reverse primer

2.4.4.3 Evaluation of primers sequences

To evaluate if the primers described in previous studies could be used to amplify the African *Bartonella* strains, the *gltA* sequences of *Bartonella* strains from Africa as well as representative *Bartonella* strains were obtained from the public domain (Genbank,NCBI) (Table 2.7). The *gltA* sequences were aligned with primer sequences listed in Table 2.6 using ClustalW (Hall, 1999).

Table 2.7: The *gltA* gene sequences of *Bartonella* species used for evaluation of primer sequences obtained from previous studies

***Bartonella* species**	**Genbank accesion number**	**Strain**	**Location**	**Host**	**Reference**
B. henselae	NC005956	Houstin - 1	USA	Human	Norman *et al*., 1995
B. bovis	EF32056	N-05-1406	France	Cattle	Birtles and Raoult, 1997
B.quintana	NC005955	Touluse	USA	Louse/Human	Birtles and Raoult, 1997
B.clarridgeiae	NC014932	Strain 73	USA	Ectoparasites	Birtles and Raoult, 1997
Bartonella spp	HM545140	M1-44	Kenya	bats	Kosoy *et al*., 2010
Bartonella spp	HM545139	M491	Kenya	bats	Kosoy *et al*., 2010

2.4.4.4. Optimization of the PCR assay for detection of *Bartonella*

PCR assay for detection of *Bartonella* was initially optimized using different parameters following a modified Taguchi method (Cobb and Clarkson, 1994). Optimization focused on $MgCl_2$, primer template and dNTP concentrations as well as the cycling conditions using the genomic DNA of *Bartonella henselae* (see section 2.2.1.4).

2.2.4.5 Conventional PCR targeting the partial *gltA* gene of *Bartonella*

Two µl (10 ng/µl) of the genomic DNA of *B. elizabethae, B. grahamii, B. henselae* and *B. vinsonii* was added to the mastermix comprising of the following: 5 µl of 10 X Dream *Taq* polymerase buffer (100 mM KCl, 100mM $(NH4)_2$ SO_4 and 20 mM $MgCl_2$) (Fermentas, USA), 5 µl of 2mM dNTP mix (Fermentas, USA), 2 µl of forward primer (10 pmol BhCS781.p) (Table 2.6) (IDT), 1 µl of reverse primer (10 pmol, BhCS1137.n) (Table 2.6) (IDT), 0.5 µl Dream *Taq* polymerase (1.25 U/µl) (Fermentas,USA) and 33.5 µl of nuclease free water (Promega, USA) to make the final of 50 µl. A no template negative control was included in this assay. Amplification was carried out in a DNA Thermo cycler (ABI-geneAmp-2720) (Applied Biosystems, Germany) under the following conditions: Initial denaturation at 94℃ fo r 5 minutes followed by 30 cycles of denaturation at 94℃ for 30 seconds, annealing at 50 ℃ for 30 seconds, extension for 72 ℃ for 1 minute and final extensions at 72℃ for 7 minutes. The PCR products were analyzed by agarose electrophoresis as described in section 2.2.1.7. The PCR

products were purified using the Wizard SV gel and PCR cleanup system (Promega, USA), according to the manufacturer's instructions (Section 2.2.1.10).

2.2.4.6 DNA sequencing of the PCR products

The PCR products were sequenced in both direction (forward and reverse) by primers listed in Table 2.6 using the ABI Prism® BigDye® Terminator Version 3.1 cycle sequencing kit (Applied Biosystems, Germany) (as described in section 2.2.1.11).

2.2.4.7 Phylogentic analysis

The sequences were edited and trimmed using the CLC Main Workbench 6 software (CLC-bio A/S, Denmark). Pairwise alignment was performed by using the CLC Main Workbench 6 (CLC-bio A/S, Denmark), in order to determine similarity between the *gltA* gene sequences of *Bartonella* spp. obtained in this study and the *gltA* gene sequences of *Bartonella* spp. available in the public domain (GenBank; NCBI).

2.2.5 Development of positive controls for the *gltA* gene of *Bartonella*

The *gltA* gene amplified in section 2.2.4.5 was cloned into a plasmid vector and the recombinant plasmid DNA was used as positive control.

2.2.5.1 Ligation of the partial *gltA* gene of *B. henselae* into a pTZ57R/T vector

The partial *gltA* gene of *B. henselae* was cloned into a pTZ57R/T vector (Fermentas, USA) by using the InsTA cloning kit (Fermentas, USA) according to the manufacturer's instructions. The ligation reactions were divided into an experimental, positive and negative control. Each ligation reaction contained the following: Three µl of pTZ57RT vector (0.156 µg/µl), 6 µl of 5X ligation buffer (20mM Tris-HCl (pH7.5), 1mM DTT, 50mM KCl and 0.1M EDTA), 1 µl of T4 DNA ligase (5 U/µl) and 16 µl of nuclease free water (Fermentas, USA) to make a final volme of 30 µl. In the positive control reaction, 4 µl of positive control PCR product (0.84 µg/µl) derived from *Pseudomonas aeruginosa* was added and in the experimental reaction, 3 µl (0.84 µg/µl) of purified PCR product (from 2.2.4.4) was added. The reaction mixtures were incubated at room temperature for 1 hour and subsequently incubated at 4℃ overnight to obtain maximum ligation.

2.2.5.2 Transformation of *E.coli* JM109 high efficiency competent cells

The competent cells were first prepared by using the TransformAid bacterial transformation kit (Fermentas, USA) following the manufacturer's instructions. Briefly, 1 ml of TransformAid™ C-medium was pre-warmed in a 37℃ shaker incubator for 20 minutes. Freshly streaked *E. coli* JM109 cells were inoculated into a 1.5 ml pre-warmed TransformAid C-medium (Fermentas, USA) and incubated for 2 hours in a 37℃ shaker. The cells were collected by centrifugation at 13 400 x *g* for 1 minute. The cells were resuspended in 300 µl of TransformAid™T-solution A (Fermentas, USA) and incubated for 5 minutes on ice. Cells were collected by centrifugation at 13 400 x *g* for 1 minute and were resuspended again in 120 µl of TransformAid™T-solution B (Fermentas, USA) and incubated for 5 minutes on ice. Overnight ligation reactions from section 2.2.5.1 were briefly centrifuged and 2 µl of each reaction was transferred to a sterile microfuge tube and incubated for 2 minutes on ice. Fifty microliters of freshly prepared *E. coli* JM 109 competent cells were transferred into the ligation reactions and incubated on ice for 5 minutes. Fifty µl of the transformed culture, 10 µl of 100mM IPTG (Sigma-Aldrich, USA) and 40 µl of X-Gal (20 mg/µl) (Promega, USA) were plated on duplicate Luria Bertani/Ampicillin (100 ng/µl) plates and incubated overnight at 37℃. The recombinants appeared as white colonies and non recombinants appeared as blue colonies. The white colonies were selected and cultured in Luria Bertani/Ampicillin (100 ng/µl) and incubated overnight in a 37 ℃ shaker incubator in order to isolate the plasmid DNA.

2.2.5.2 Isolation of plasmid DNA

The plasmid DNA was isolated using a Gene-Jet plasmid miniprep kit (Fermentas) according to the manufacturer's instructions (as described in section 2.2.2.3).

2.2.5.3 DNA sequencing of the plasmid DNA

The plasmid DNA was sequenced as described in section 2.2.2.4. The sequences were edited and trimmed using the CLC Main Workbench 6 (CLC bio A/S, Denmark). The pairwise alignment was performed by using the Bioedit v 7.1.3 software (Hall, 1999) in order to determine similarity between the *gltA* sequence from the plasmid and the *gltA* gene sequence of *B. henselae* obtained in section 2.2.4.7

2.2.6 Detection limit of the conventional PCR assay targeting the partial *gltA* gene of *Bartonella*

The detection limit of the PCR assay targeting the partial *gltA* gene of *Bartonella* was determined by using a method as described in section 2.2.3. The initial concentration of the undiluted plasmid DNA that was determined with the NanoDrop^{TM} 1000 spectrophotometer (Fermentas, USA) was 145.8 ng/µl and this corresponded to 3.58e-8 copies/µl. The cycling conditions used in this case were described in section 2.2.4.5. A no template negative control was included.

2.3 Results

2.3.1 *Rickettsia*

2.3.1.1 Evaluation of the primers from previous studies targeting the partial *gltA* and *16S rRNA* gene of *Rickettsia*

Primer sequences obtained from previous studies were aligned on representative sequences of the partial *gltA* (Figure 2.1) and the *16S rRNA* gene (Figure 2.2) of *Rickettsia* species. The panrick2 primer sequence had one mismatch at the 6th base residue with the partial *gltA* gene *Rickettsia felis* at position 566. PanRick7 primer sequence had 3 mismatches at the 10th and 12th and 22nd base residue with the partial *gltA* gene of *Rickettsia africae, Rickettsia prowazekii* and *Rickettsia felis* at position 586 and 588 and 600, respectively. PanRick 2b primer sequence had only 1 mismatch at the 6th base residue with the partial *gltA* gene of *Rickettsia prowazekii* at position 611. NgltF primer sequence had one mismatch at the 2nd base residue with the partial *gltA* gene of *R. prowazekii, R. felis* and *R. felis* at position 865. The second mismatch of this primer was at position 880 of the *gltA* gene of *R. prowazekii.* NgltR primer sequence had two mismatches at the 2nd and 4th base residue with the partial *gltA* gene of *R. prowazekii* and *R. aeschlamannii* at position 1027 and 1030, respectively. RpCs1258 primer sequence had one mismatch at the 4th base residue with the partial *gltA* gene of *R. prowazekii* at position 1159. The second mismatch occurred at position 1164 with all the *gltA* gene sequences used in this analysis. The 16SF primer sequence had one mismatch with all the representative *Rickettsia* sequences at the 2nd base residue at position 659 of the gene. The 16SR primer sequence had 2 mismatches at the 4th and 20th base residue with the partial *16S rRNA* gene sequence of *R. prowazekii* at position 1085 and 1100, respectively. Although, novel *Rickettsia*

species might not be detected with these primers, the alignments in Figures 2.1 and 2.2 indicate that the published primers should detect the partial *gltA* and the*16S rRNA* genes of *Rickettsia* species reported in Africa.

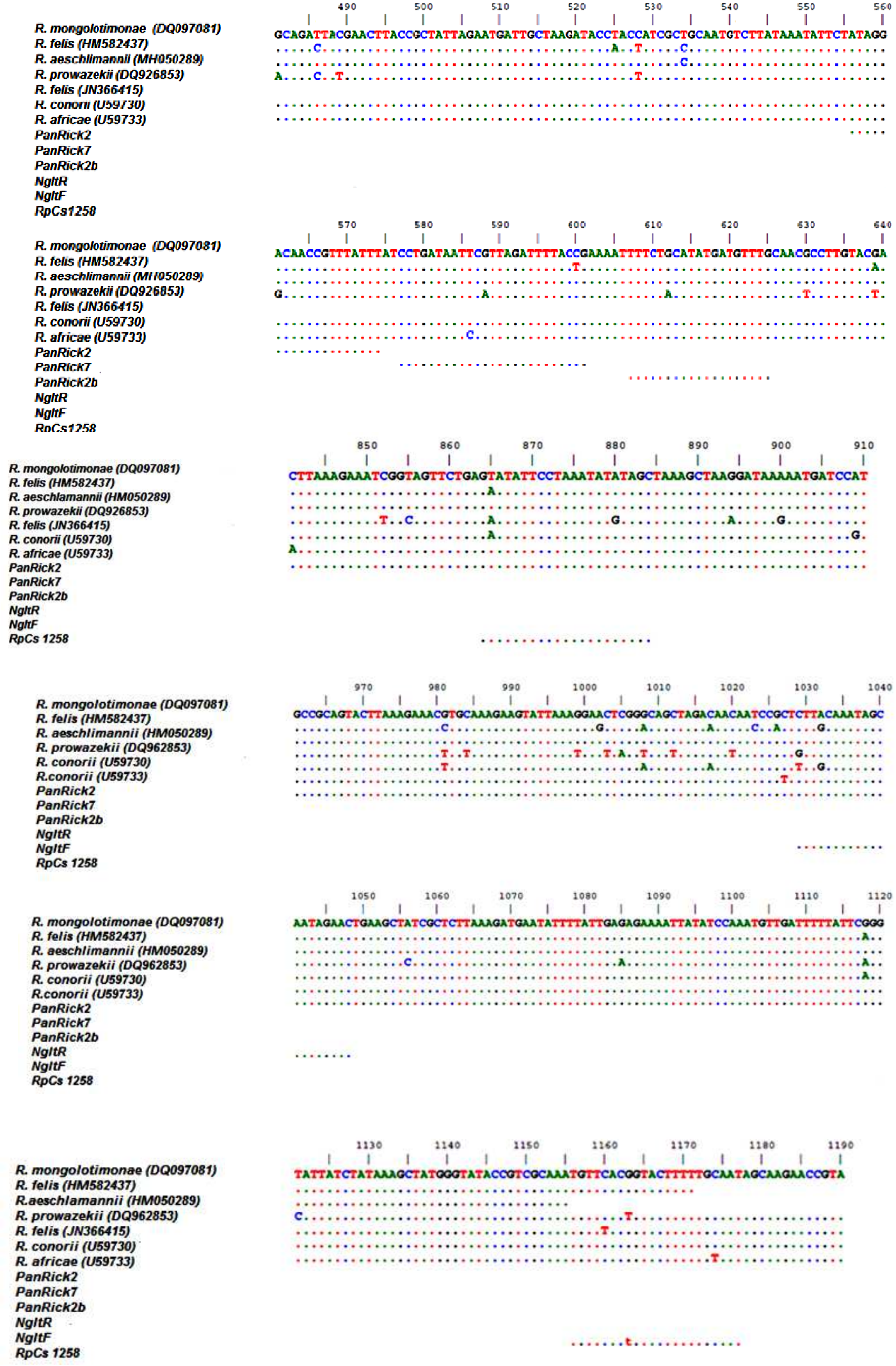


Figure 2.1: Multiple alignment of representative African *Rickettsia* species indicating the primer binding site on the *gltA* gene. The dots indicate identical base pairs

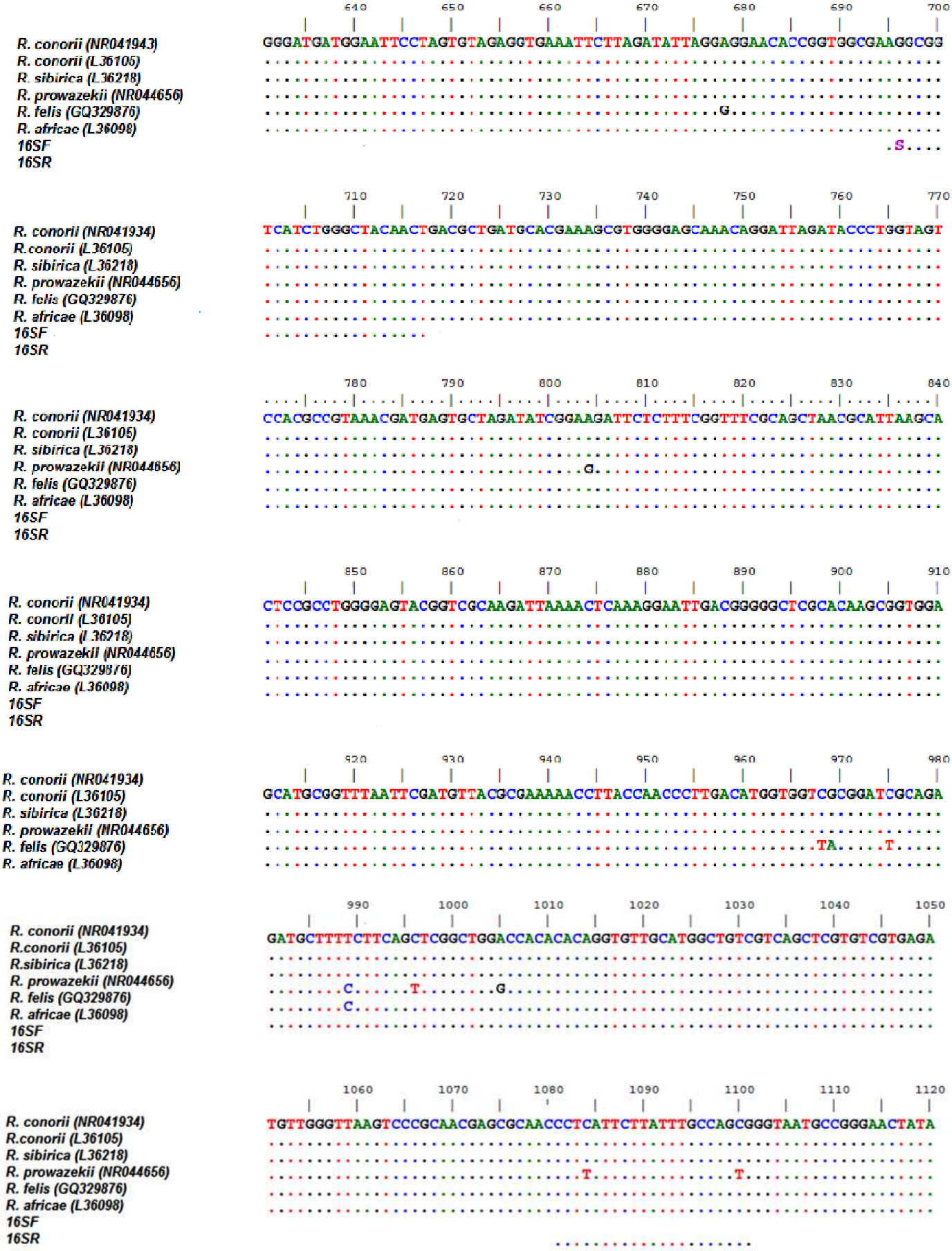


Figure 2.2: Multiple alignment of representative African *Rickettsia* species representing the primer binding site on the *16S rRNA* gene. The dots indicate identical base pairs.

2.3.1.2 Optimization of the PCR assays for detection of *Rickettsia*

All the PCR assays were optimal with respect to primer concentration at 10 pmol primer per reaction. Conventional and nested PCR targeting the partial *gltA* gene, and the conventional PCR targeting the partial *16S rRNA* was all optimal at the template concentration of 20 ng/µl. The real time PCR was optimal at a low template concentration (5ng/µl) when compared to the other assays. The optimal conditions are summarized in Table 2.8.

Table 2.8: Optimal PCR conditions for the detection of the partial *gltA* and *16S rRNA* gene of *Rickettsia*

PCR		**Parameters**					
	Partial gene targeted	**[Template](ng/µl)**	**[$MgCl_2$] (mM)**	**[Primer](pmol)**	**Annealling tempature (℃)**	**[Probe] (pmol)**	**[dNTP] (mM)**
Real time	*gltA*	5	2	10	40	4	0.8
Conventional		20	2	10	50	-	10
Nested		20	2	10	42	-	10
Conventional	*16S rRNA*	20	2	10	55	-	10

- indicates not tested

2.3.1.3 Amplification of the partial *gltA* gene of *Rickettsia conorii* by real time PCR

The partial *gltA* gene was amplified from the extracted genomic DNA of *Rickettsia conorii*. Amplification was achieved after the 25^{th} cycle (Figure 2.3)

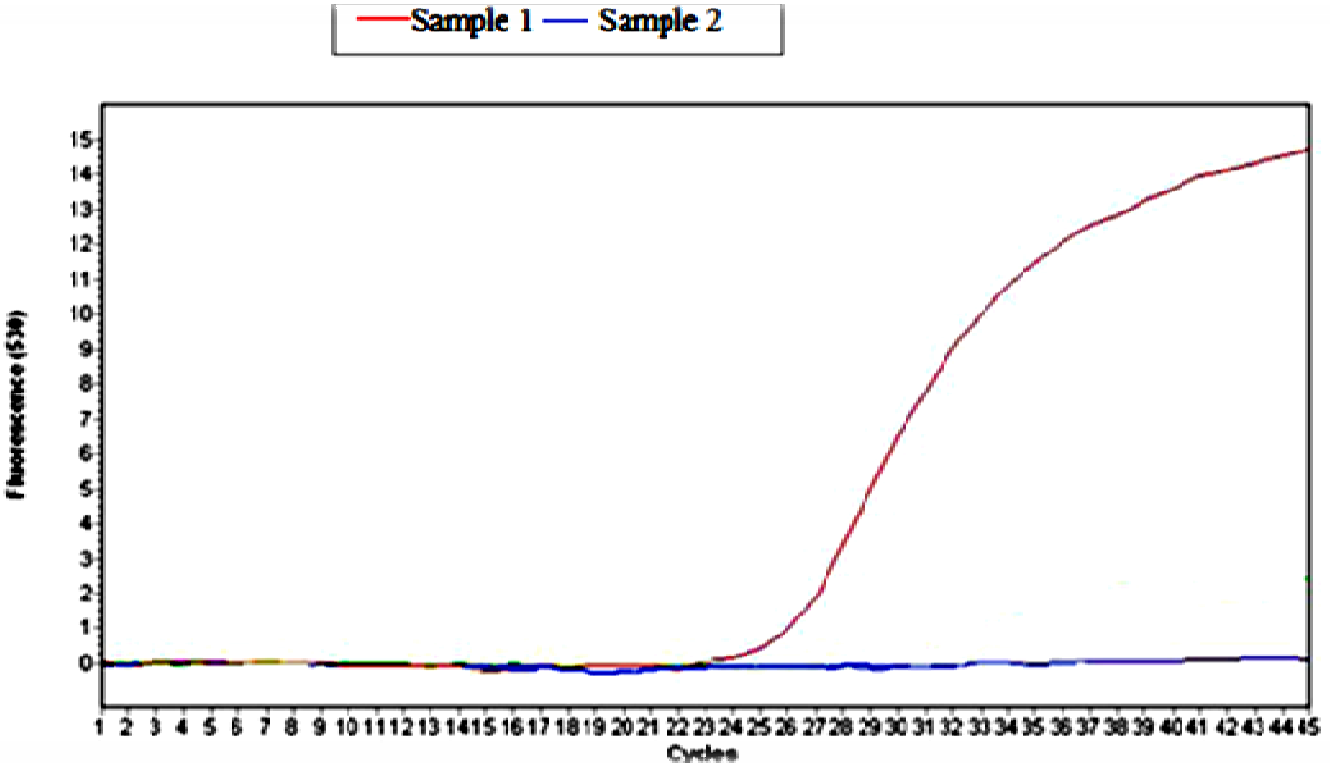


Figure 2.3: The real time PCR amplification curve generated during the amplification of the partial *gltA* gene of *Rickettsia conorii*. Sample 1*: R. conorii* DNA, Sample 2: No template negative control.

2.3.1.4 Conventional PCR and sequencing of the partial *gltA* and *16S rRNA* genes of *Rickettsia conorii*

The partial *gltA* and *16S rRNA* gene of *R.conorii* were amplified by conventional and nested PCR (Figure 2.4). The *gltA* gene PCR products were 645 bp and 202 bp in size for conventional and nested PCR, repectively and the *16S rRNA* PCR product was 400 bp. The DNA sequences generated shared a 99% similarity with the *gltA* gene of *Rickettsia conorii* stain malish 7 (NC 003101.1) and a 99% with the *16S rRNA Rickettsia conorii* strain Malish 7 (L36107).

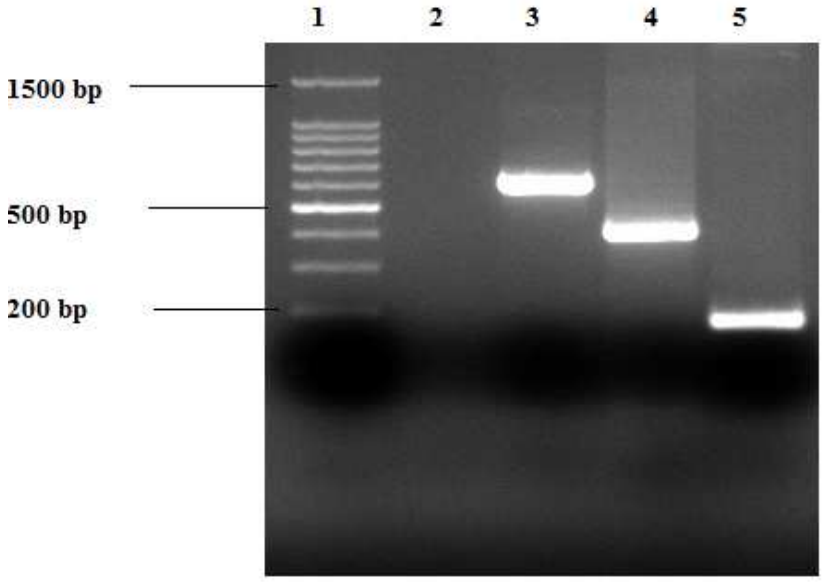


Figure 2.4: Agarose gel electrophoresis analysis illustrating the partial *16S rRNA* (400 bp) and *gltA* (645 and 202 bp) genes of *Rickettsia conorii* amplified during conventional and nested PCR, respectively. Lane 1: 100bp DNA ladder (Fermentas), Lane 2: No template negative control, Lane 3: Conventional PCR targeting the partial *gltA* gene of *R. conorii*, Lane 4: Conventional PCR targeting the partial *16S rRNA* gene of *R. conorii*, Lane 5: Nested PCR targetting the partial *gltA* gene of *R. conorii*.

2.3.1.5 Generation of positive controls for the partial *gltA* and *16S rRNA* gene of *Rickettsia*

The partial *16S rRNA* (400bp) and *gltA* (202 and 645bp) generated with PCR and were cloned into a pGEM-T-Easy vector. The cloned partial genes had 100% similarity with the partial *16S rRNA* and *gltA* gene sequences of *R. conorii* in section 2.2.1.12 (Figure 2.5 and 2.6). Recombinant plasmid DNA generated was used as positive contol DNA for the PCR assay of the gene of interest. The real time product could not be cloned because the fragment was very small. However, the recombinant plasmid DNA of the partial *gltA* gene for conventional PCR was used as the positive control for the real time PCR since the target sequence (70 bp) was also present in the plasmid control (Figure 2.5).

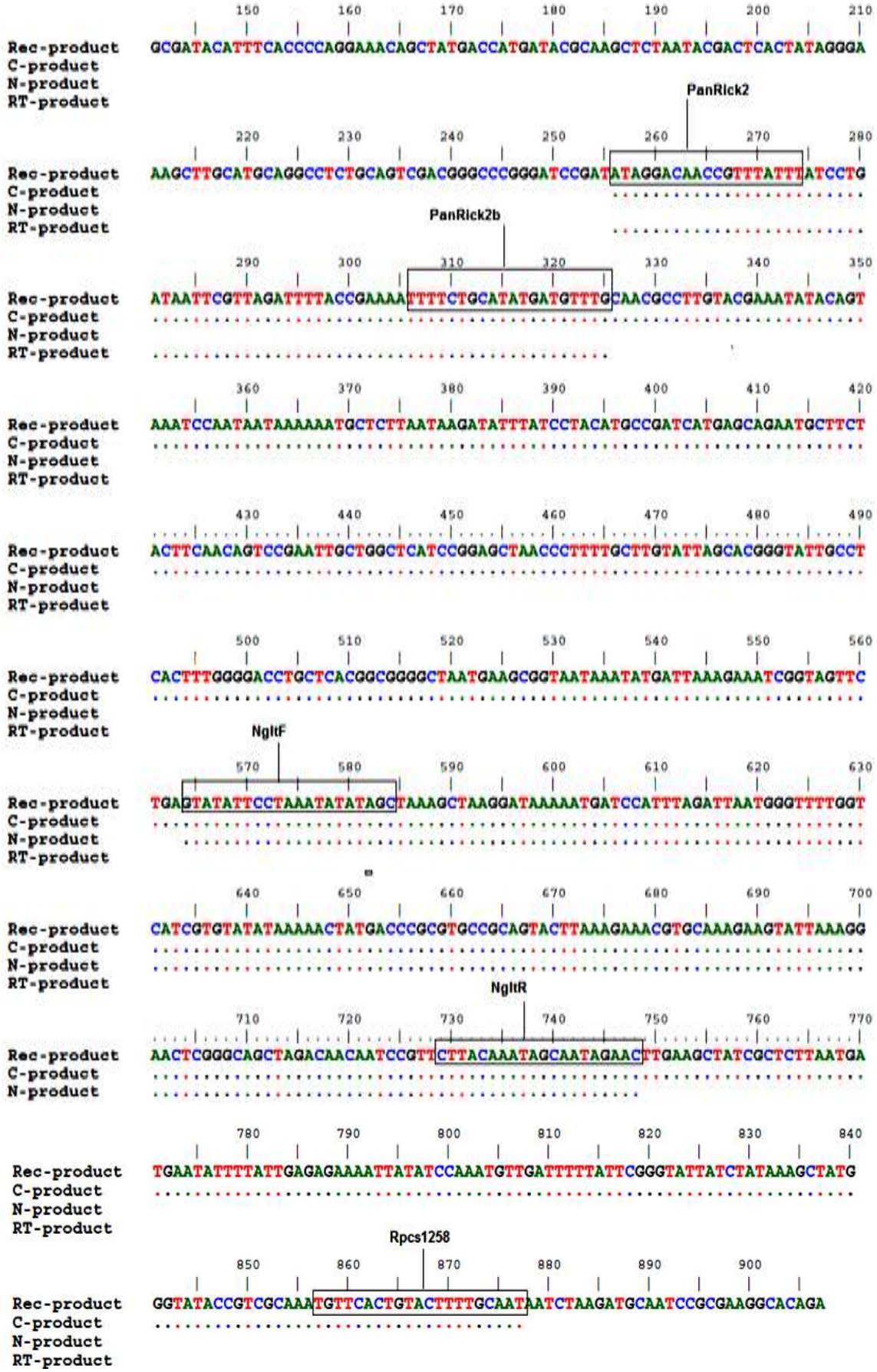


Figure 2.5: Pairwise alignment analysis of the partial *gltA* gene of *Rickettsia conorii* with the recombinant plasmid DNA containing partial *gltA* gene of interest. C-product: Conventional PCR product of the partial *gltA* gene (645 bp), Rec-product: Recombinant plasmid DNA containg the partial *gltA* gene of interest, N-product: nested PCR product of the partial *gltA* gene (202 bp), RT- product: Real time PCR product of the partial *gltA* gene (70bp). The colored dots indicate identical base pairs

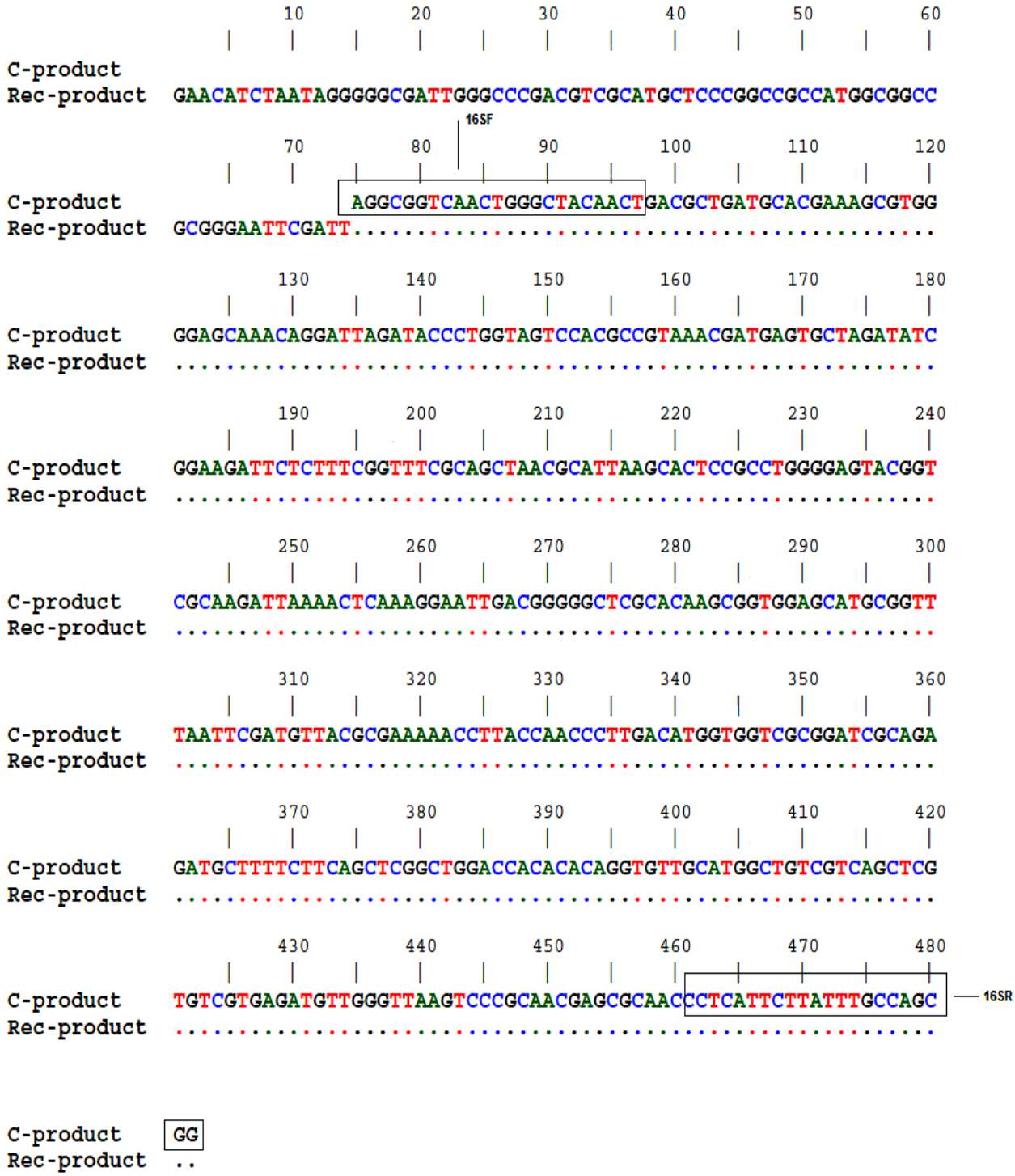


Figure 2.6: Pairwise alignment analysis of the partial *16S rRNA* gene of *Rickettsia conorii* with the recombinant plasmid containing partial *16S rRNA* gene. C-product: Conventional PCR product of the partial *16S rRNA* gene, Rec-plasmid: Recombinant plasmid DNA containing the partial *16S rRNA* gene. The coloured dots indicate identical base pairs.

2.3.1.6 Sensitivity of the PCR assays for detection of *Rickettsia*

Recombinant plasmid for the partial *gltA* and *16S rRNA* were generated and were successfully used to determine the detection limit of the PCR assays for detection of *Rickettsia*. Among the assays, the real time PCR targeting the partial *gltA* gene showed the highest detection limit and was able to detect up to 1 copy of the target (Table 2.9) and the conventional PCR targeting the partial *gltA* and *16S rRNA* showed the least (detected up to 10^3 copies) (Figure 2.7B and C) The nested PCR was able to detect up to a 100 copies of the target gene (Figure 2.7A)

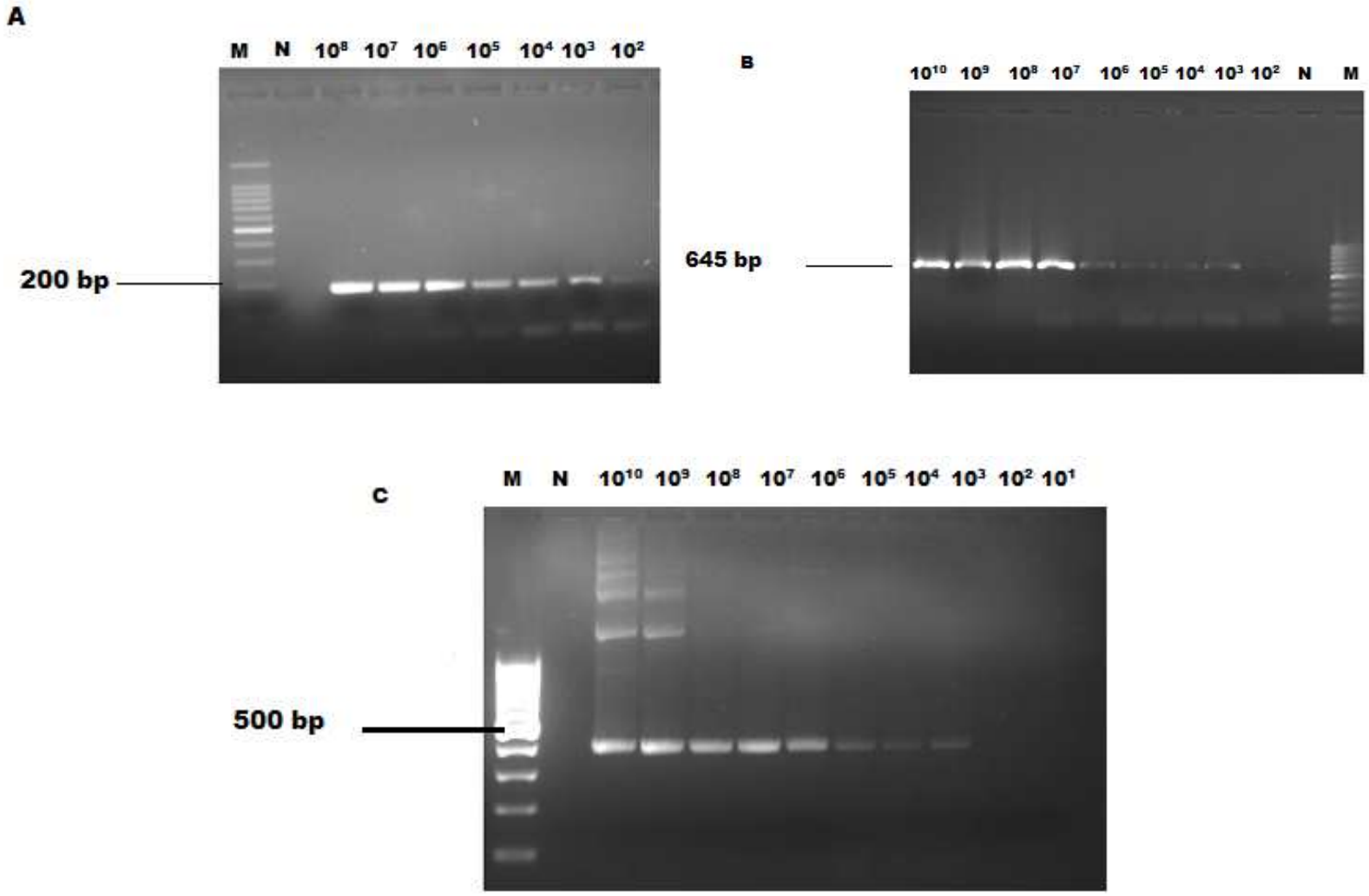


Figure 2.7: Agarose gel electrophoresis analysis illustrating the copies of the partial *gltA* and *16S rRNA* genes of *Rickettsia conorii* that were generated in different PCRs when the recombinant plasmid DNA is serially diluted (10^{-1} to 10^{-10}). A: Copies of the *partial gltA* gene (200bp) of *R. conorii* generated in nested PCR, B: Copies of the partial *gltA* (645 bp) of *R. conorii* generated in a conventional PCR. C: Copies of the partial the *16S rRNA* of *R. conorrii generated* in conventional PCR. M-100bp DNA ladder (Fermentas, USA), N: No template negative control. The values at each lane indicate the copy number of the respective genes.

Table 2.9: Real time PCR analysis obtained after amplification of different dilutions of the recombinant plasmid containing the partial *gltA* gene of *Rickettsia conorii*

Source of plasmid	**Concentration Of plasmid (g/µl)**	**Copies of the *gltA/16S rRNA* gene sequence**	**Cp values**	**Mean**	**Standard deviation**	**Coefficient variation**
Stock	217.5e-11	NA	N/A	N/A	N/A	N/A
Dilution 1	8.2e-9	10^9	8.79 8.68 8.69	8.69	0.02	0.23
Dilution 2	8.2e-10	10^8	11.31 11.83 11.10	11.41	0.37	3.22
Dilution 3	8.2e-11	10^7	14.80 14.98 14.55	14.78	0.22	1.48
Dilution 4	8.26e-12	10^6	17.99 18.00 17.70	17.90	0.17	0.949
Dilution 5	8.2e-13	10^5	21.93 21.98 22.19	22.03	0.13	1.36
Dilution 6	8.2e-14	10^4	23.91 24.92 24.87	24.566	0.569	2.32
Dilution 7	8.2e-15	10^3	26.23 26.77 26.28	26.43	0.3	1.13
Dilution 8	8.2e-16	10^2	27.15 27.29	27.22	0.10	0.366
Dilution 9	8.2e-17	10	28.56 28.62	28.59	0.04	1.34
Dilution 10	8.2e-18	1	33.42 33.52	33.47	0.07	0.2

CV=SD/Mean X100 , NA -indicates not performed

2.3.1.7 Real time PCR statistics

The partial *gltA* gene (70bp) of *Rickettsia conorii* was amplified in all the dilution factors (10^{-1}-10^{-11}) representing 1 to 10^{10} copies of the partial *gltA* gene. The crossing point (Cp) values were determined by the second derivative maximum method function of the Lightcycler ®software version 4.05 (Section 2.3.1.2). A no template negative control was also included in the analysis and as was expected it was not amplified. To determine the effeciency of this assay, standard curve was constructed by ploting the Cp values versus the log concentration of the target (Figure 2.8). The PCR efficiency was 1.972 (approximately 97%), the error rate was 0.173 and the slope was -3.56, respectively. To determine the intra assay variations (precision), the Cp values obtained were evaluated with regards to the standard deviation (SD) and coefficient variation (CV). The CV values obtained were 0.7 to 3.22 which is in the recommended range of 2-4% (Pfaffl *et al*., 2002) (Table 2.9).

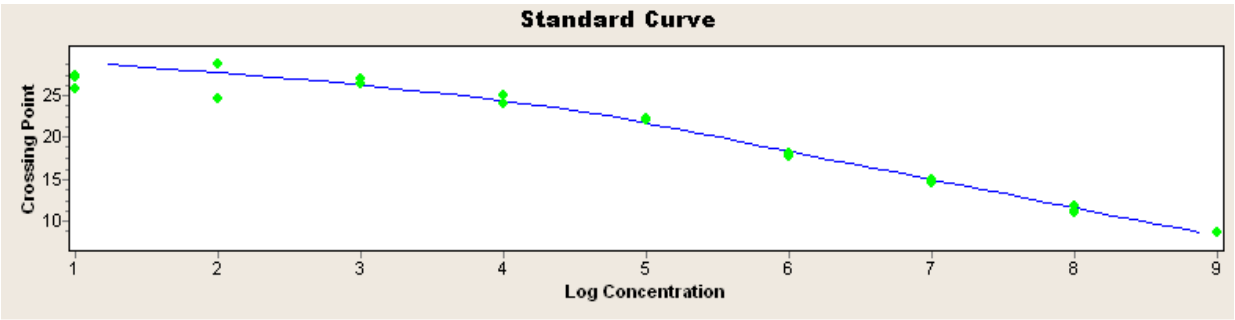


Figure 2.8: Standard curve constructed by plotting the Cp values against the log concentration of the serially diluted recombinant plasmid containing the partial *gltA* gene amplified in real time PCR. The reaction was performed in itriplicates

2.3.2 *Bartonella*

2.3.2.1 Evaluation of primer binding site on gltA gene of *Bartonella* species

The primers were aligned on the *gltA* gene sequences of *Bartonella* species. BhCs 781.p primer sequences had two mismatches at the 9th-11th base residues with the *gltA* gene sequence of *Bartonella* strain from Kenya corresponding to 791-793, respectively. BhCs 1137.n primer sequences had one mismatch at the 14th base residue corresponding to position 1152 of *Bartonella clarridgeiae* (Figure 2.9)

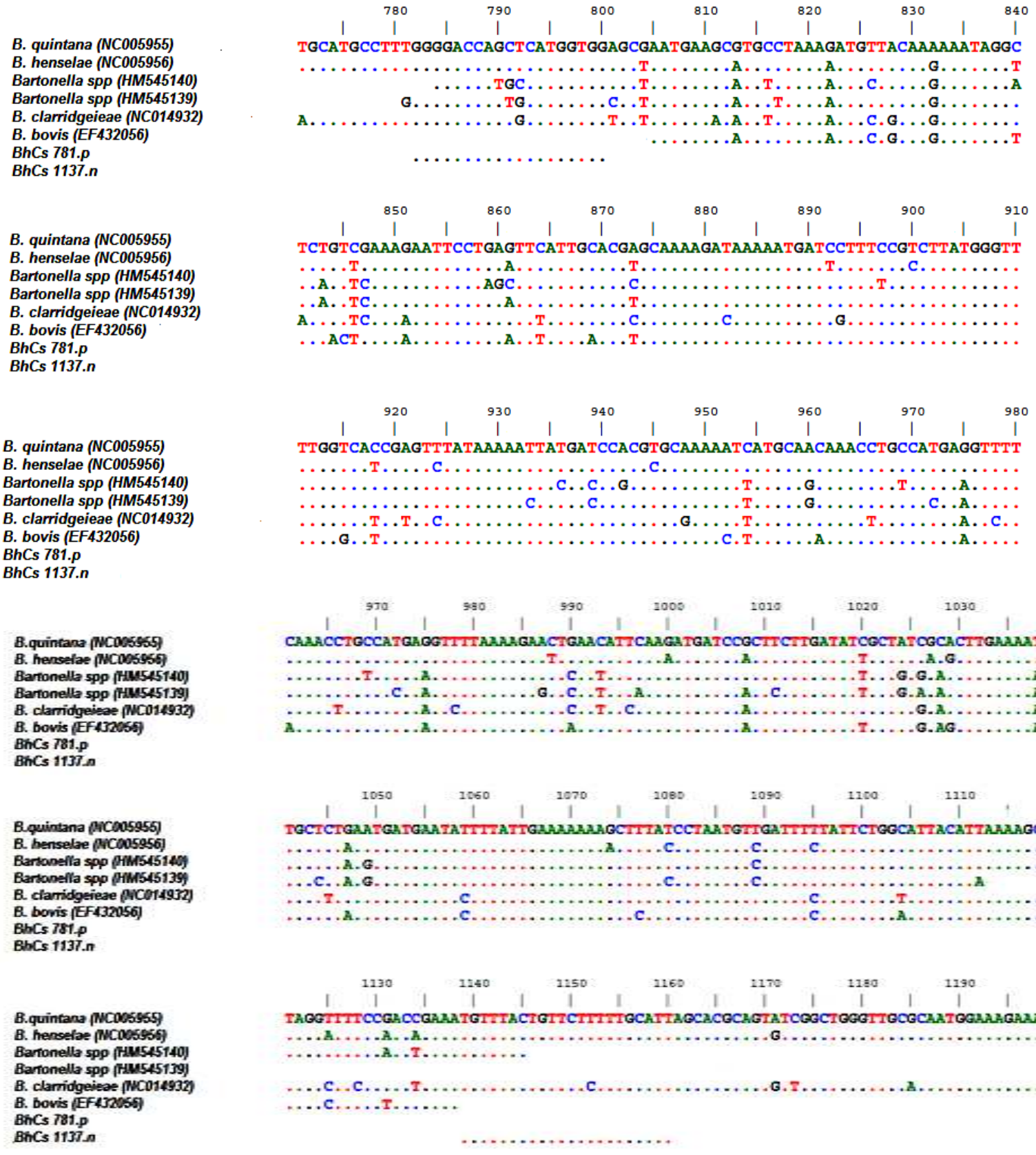


Figure 2.9: Multiple alignment of representative sequences of *Bartonella* species indicating the primer binding site on the *gltA* gene. The dots indicate identical base pairs

2.3.2.2. Optimization of the PCR assay for detection of *Bartonella*

The conventional PCR targeting the partial *gltA* gene was optimized with the final conditions at 10 pmol primer, 10mM dNTP, 2mM $MgCl_2$, 20 ng/µl templates and a 50℃ annealing temperature.

2.3.2.3 Amplification and sequencing of the partial *gltA* gene of *Bartonella*

The partial fragment of the *gltA* gene was amplified and yielded a 379 bp PCR product (Figure 2.10). The DNA sequence generated from *B. henselae* shared a 99% similarity with the *gltA* sequence of *Bartonella henselae* strain M8SHD (FJ492803.1), the DNA sequence generated from *B. grahamii* shared a 98% similarity with the *DNA* sequence of *Bartonella grahamii as4up* (CP001562.1), the *gltA* sequence generated from *B. vinsonii* shared 98% with the *gltA* sequence of *Bartonella vinsonii subsp berkhoffi* (AF143445.1) and finally the DNA sequence generated from *B. elizabethae* shared a 99% with the *gltA* sequence of *B. elizabethae* strain BR03 (GU056193.1).

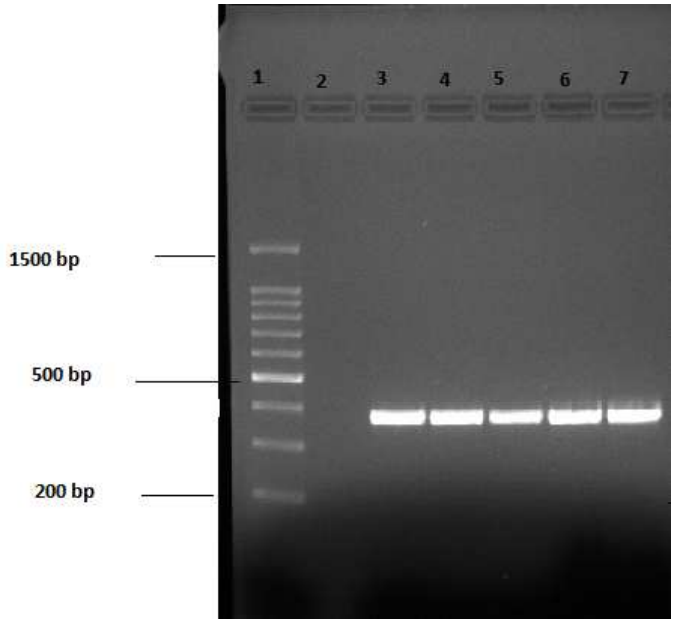


Figure 2.10: Agarose gel electrophoresis indicating the partial fragment of the *gltA* gene of *Bartonella* spp. amplified using conventional PCR. Lane 1: 100bp DNA ladder (Fermentas), Lane 2: No template negative control, Lane 3: Partial *gltA* gene of *B. henselae*, Lane 4: Partial *gltA* gene of *B. quintana*, Lane 5*:* Partial *gltA* gene of *B. vinsonii*, Lane 6: Partial *gltA* gene of *B. grahammi*, Lane 7: Partial *gltA* gene of *B. elizabethae*

2.3.3 Generation of positive control DNA of *Bartonella*

The partial citrate synthase gene (*gltA*) of *Bartonella henselae* was cloned into the pTZ57R vector. After sequence analyses the cloned partial sequence of the *gltA* was confirmed to be similar to *B. henselae* (Section 2.3.2.3) (Figure 2.11). The recombinant DNA generated was used as a positive control for the PCR assay.

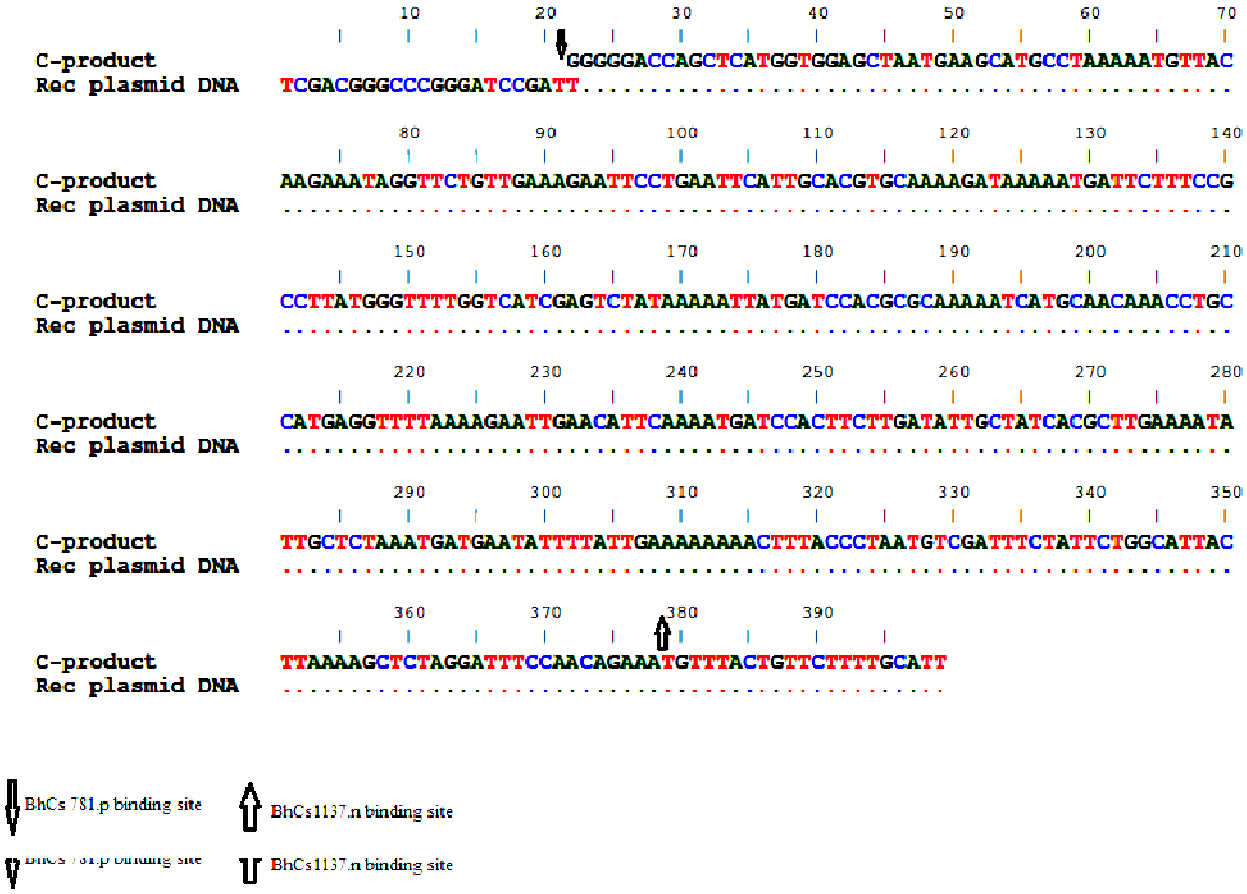


Figure 2.11: Pairwise alignment analysis of the partial *gltA* gene of *Bartonella henselae* with the recombinant plasmid containing partial *gltA* gene of *B. henselae*. C-product: Conventional PCR product of the partial *gltA* gene, Rec-plasmid: Recombinant plasmid DNA containing the partial *gltA* gene. The coloured dots indicate identical base pairs

2.3.4 Sensitivity of the PCR assay for the detection of *Bartonella*

The partial fragment of the *gltA* of *Bartonella* was successfully cloned into the pTZ57RT vector and successfully used to determine the detection limit of the assay. The assay was able to detect up to 10^4 copies of the partial g*ltA* gene. A no template negative control was also included in the analysis and as expected it was not amplified (Figure 2.12).

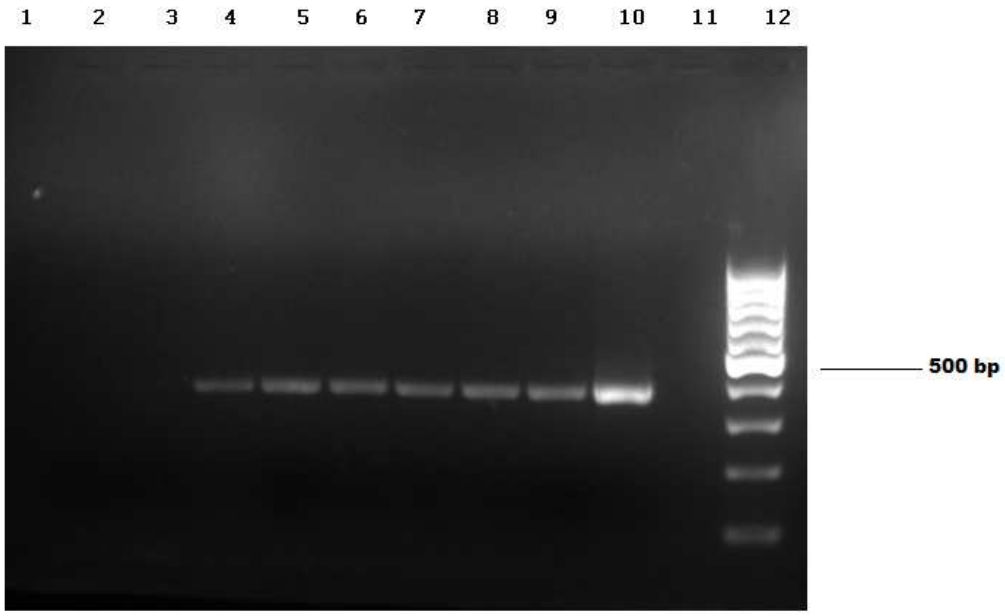


Figure 2.12: Agarose gel electrophoresis indicating the copies of the partial the *gltA* gene of *Bartonella* generated during conventional PCR by using the 10- fold serial dilutions of the recombinant plasmid DNA. Lane 1: 10^1 copies, Lane 2: 10^2 copies, Lane 3: 10^3copies, Lane 4: 10^4copies, Lane 5: 10^5 copies, Lane 6: 10^6 copies, Lane 7: 10^7 copies, Lane8: 10^8 copies, Lane 9:10^9copies, Lane 10:10^{10} copies, Lane 11: No template negative control, Lane12: 100 bp DNA ladder (Fermentas, USA)

2.4 Discussion

The polymerase chain reaction offers advantages over other methods used for detection of *Rickettsia* and *Bartonella* in that it is rapid, sensitive and bacterial DNA can be amplified directly from clinical specimens and characterized by DNA sequencing (Agan *et al*., 2002; Wölfel *et al.,* 2008). In this study, we evaluated the different PCR assays targeting the *gltA* and *16S rRNA* genes of *Rickettsia.* The *16S rRNA gene* was selected for our assays because this gene is conserved in the genus *Rickettsia,* and therefore it can be assumed that all *Rickettsia* species reported in this area will be detected when targeting this gene. Furthermore, the sequences of the *gltA* gene are variable and can be used to differentiate closely related *Rickettsia* species (Fournier *et al.,* 2003; Fournier and Roault, 2009). For the detection of *Bartonella*, the conventional PCR assay targeting the partial *gltA* gene was evaluated. This gene has proven to be a useful tool for detection of *Bartonella* and it was recently used to detect *Bartonella* DNA in bats from Kenya, Guatemala and Taiwan *(*Kosoy *et al*., 2010; Bai *et al*., 2011, Lin *et al.,* 2011). This gene is not only conserved in the genus *Bartonella* but has been previously used to identify *Bartonella* to the species level (Zeaiter *et al*., 2002).

Primer sequences for the *gltA* and *16S rRNA* genes of *Rickettsia* and *Bartonella* were obtained from previous studies and evaluated to ensure that they could amplify *Bartonella* and *Rickettsia* species/strains from Africa. All the primers sequences were aligned with the representative sequences from the public domain (NCBI). There were a few mismatches at the 5'end terminus and in the middle of the primers. Although, novel *Rickettsia* and *Bartonella* species might not be detected with these primers, the alignments in Figure 2.1, 2.2 and 2.9 indicate in theory that the published primers will be able to detect the partial *gltA* and the *16S rRNA* genes of *Rickettsia and Bartonella* species reported in Africa. These genes (*16S rRNA* and *gltA*) are highly conserved within the genus *Rickettsia* and *Bartonella* and as such, chances of these primers not detecting other strains that have never been reported in this region are less (Zeaiter *et al*., 2003; Fournier and Raoult, 2009).

The assays for detection of *Rickettsia* and *Bartonella* DNA were initially optimized using the Taguchi's method of optimization (Corb and Clarkson. 20003). The PCR assays were further compared with those developed from previous studies. Our real time PCR assay had a lower annealing temperature (40℃) when compared to 55℃ used in Wölfel *et al.,* (2008) study. Weighardt *et al*., (1993) reported that the specificity of the PCR assay is reduced when a low annealing temperature is used but we did sequence all positive reactions and would have detected false negatives. The sensitivity of our real time PCR assay was higher than the assay developed Wölfel *et al*., (2008). Our assay was able to detect up to 1 copy of the target gene when compared to 12.3 copies detected in Wölfel *et al*., 2008. The quencher that was used in Wölfel *et al.,* (2008) was tetramethylrhodamine (TMR), whereas in our study a black hole quencher (BHQ) was used. BHQs are advantageous when compared to TMRs because they do not emit fluorescence thereby decreasing background fluorescence (Drake and Tan, 2004; Ge, 2012). TMRs have their own emission wavelength that can contribute to the fluorescent signal (Drake and Tan, 2004, Ge, 2012).

The PCR conditions and the sensitivity of conventional PCR assays targeting the partial *gltA* and *16S rRNA* genes of *Rickettsia* was the same as the PCR assays developed from previous studies (Perotti *et al*., 2006; Wölfel *et al*., 2008). The sensitivity of our nested PCR targeting the *gltA* gene of *Rickettsia* was compartible to the other nested PCR assay developed for detection of *Rickettsia* (Wiksow *et al*., 2007; Prakash *et al.,* 2011). The conditions used in our conventional PCR targeting the partial *gltA* gene of *Bartonella* were the same as those from Kosoy *et al*., (2010). The sensitivity of this assay was low (detected up to 10^4 copies), but it was similar to what was observed in previous studies (Norman *et al.,* 1995; Kosoy *et al.,* 2010).

The real time PCR assay targeting the partial *gltA* of *Rickettsia* showed the highest detection limit and as such it was used as the initial screening assay for detection of rickettsial DNA in our specimens (bat blood samples). Although, the real time PCR had the highest sensitivity, the product targeted was very small and as a result it was difficult to obtain a usable sequence from this fragment. For this reason conventional and nested PCR were used to generate longer products that could be sequenced. *Rickettsia* replicates in the endothelial cells and generally are found in low concentrations in the blood (Parola and Raoult, 2007). Parola *et al.,* (2005) reported

that the conventional PCR has poor sensitivity for detecting *Rickettsia* in blood specimens. To increase sensitivity, a nested PCR was adopted and was able to detect up to 10^2 copies of the *gltA* gene of *Rickettsia*. The sensitivity of the assay for detection of *Bartonella* DNA was also evaluated. The detection limit of this assay was low (detected up to 10^4 copies), however, *Bartonella* replicates in the erythrocytes of mammals (Breitschwerdt *et al.,* 2010), and recent studies by Kosoy *et al*., (2010) and Bai *et al.,* (2011) showed that significant amount of *Bartonella* DNA can be detected in bat blood samples using this assay.

The partial genes of *Rickettsia* (*gltA* and *16S rRNA*) and *Bartonella* (*gltA* gene) were cloned into plasmid vectors and were used as positive control DNA for the assays. DNA may not be always available because *Rickettsia* and *Bartonella* are difficult to isolate in culture and isolations require longer incubation periods. *Rickettsia* and *Bartonella* are pathogenic and/or require special environments such as biosafety 3 laboratory which is not always available (Anderson and Neuman, 1997; La Scola and Raoult, 1997; Welch and Slater, 1999; Agan *et al.,* 2002; Dworkin *et al.,* 2006; Wölfel *et al.,* 2008). Furthermore, the genomic DNA will not have to be extracted each time the PCR reaction is performed. Cloning the partial genes into the plasmid vector ensures that they can remain stable for longer periods.

PCR assay for detection of *Rickettsia* and *Bartonella* were evaluated and optimized and synthetic controls for the assays were generated. These assays will be used in future as surveillance tools for these pathogens in blood samples collected from bats.

Chapter 3: Detection of rickettsial DNA in southern African bats

3.1 Introduction

Rickettsias are obligate intracellular symbionts of eukaryotes and are transmitted to vertebrate hosts by blood-feeding arthropods that act as the reservoir (Merhej and Raoult, 2011). This group of bacteria are mostly noted for causing human diseases including Rocky Mountain spotted fever, Mediterranean spotted fever and epidemic typhus (Weinert *et al.,* 2009). At least six rickettsial species have been implicated in human infections in Africa (Parola and Raoult, 2001; Hendershot and Sexton, 2009), however, the most frequent rickettsial-borne disease in South Africa is tick bite fever caused by *R. conorii* and *R. africae* respectively (Frean *et al*., 2008). In addition to these rickettsial species, *R. sibirica mongolotimonae* and *R. aechlamannii* have been reported in this region (Pretorius and Birtles, 2002; Pretorius *et al.,* 2004b).

Rickettsia have been reported in a wide variety of other hosts including dogs (Harrus *et al*., 2007), cats (*Kelly et al.,* 2010) and rodents (Choi and Lee, 1996), but have never been detected in bats. Previous studies by Steinhaus (1947) showed experimentally that bats are susceptible to *Rickettsia rickettsii* (agent of RMSF) infection. Rickettsiosis in bats has been poorly investigated and currently there are only two studies available that have investigated rickettsial infections in bats. The first study in Georgia (USA), sera from 56 *Eptesicus fuscus* (insectivorous bats) were tested against *R. rickettsii* and *R. conorii* antigens. Sero-conversion was obtained against *R. rickettsii* antigen in 1.8% (1/56) *Eptesicus fuscus* tested, but rickettsial DNA was not detected (Reeves *et al*., 2006a). In another study in Brazil, bats from *Molossidae*, *Vespertilionidae and Phyllostomidae* families were screened for antibodies against rickettsial antigens. Overall sero-conversion was 8.6% (39/451), 9.5 %(34/358), 7.8% (28/358) and 1.1% (4/358) against *R. rickettsii, R. parkeri*, *R. amblyommi* and *R. rhipicephali*, respectively (D Áuria *et al*., 2010) and they did not attempt to detect rickettsial DNA.

Bats mobility, broad distribution, social behaviours (communal roosting), long life span and ectoparasites infestation make them ideal reservoirs and sources of infections of various etiological agents including *Rickettsia*. The occupation of some of the bat species in man-made structures could bring a closer association with species with humans (Wong *et al.,* 2007). A study by Loftis *et al.,* (2005), reported

transovarial maintenance of spotted fever *Rickettsia* in bat ectoparasites (*Carios kelleyi*). These ticks infest bats that live close to human buildings/dwelling and can feed on human blood and cause infections (Gill *et al*., 2004). Subsequent studies by Reeves *et al.,* (2006a) showed that these ticks (*Carios kelleyii*), can transmit *Rickettsia* pathogens to susceptible hosts during a blood meal. Although, transmission of *Rickettsia* by bat ectoparasites to humans has never been investigated, these findings suggest potential transmission.

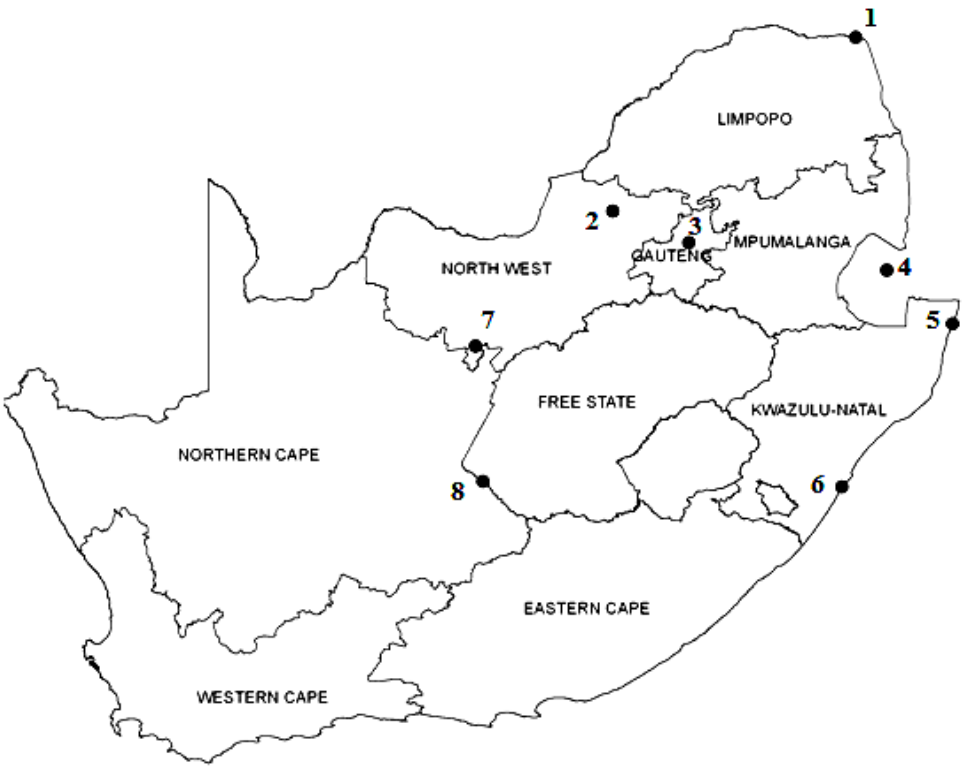
Surveillance of *Rickettsia* in different hosts is based on serological testing (IFA and ELISA) and conventional PCR targeting the *gltA*, *ompA, ompB*, and *16S rRNA* genes (Loftis *et al.,* 2005; Reeves *et al.,* 2006a; D Auria *et al.,* 2010). Numerous studies have reported that available serological tests lack specificity (La Scola *et al.,* 1997; Raoult and Roux, 1997; Wölfel *et al*., 2008) and the sensitivity of conventional PCR assays is low when detecting *Rickettsia* in blood specimens (Fenollar *et al*., 1999; Parola *et al.,* 2005). Nevertheless, sensitive and specific rapid assays have been developed to complement these existing techniques. These include quantitative real time and nested PCR assay targeting several rickettsial genes (Stenos et a*l.,* 2005; Svraka *et al.,* 2006; Wölfel *et al.,* 2008).

The aim of this chapter was to identify *Rickettsia* species circulating in southern African bat species. Bat blood samples were collected from different locations in South Africa and Swaziland as part of an ongoing lyssavirus and other zoonotic pathogen surveillance research program. Genomic DNA was extracted from bat blood samples and nucleic acid detection methods evaluated in chapter 2, were used to amplify the conserved *16S rRNA* and the variable *gltA* gene of *Rickettsia*. The amplicons were sequenced and phylogenetic relationships were determined. This study reported for the first time the detection of *Rickettsia* DNA in South African and Swaziland bat species.

3.2 Materials and methods

3.2.1 Bat blood samples used in this study

Bat blood samples were collected from different locations in South Africa and Swaziland as part of an ongoing lyssavirus and other zoonotic agents' surveillance research program in bats. A total of 354 blood samples were collected between 2007 and 2010 from the Kruger National Park (Pafuri: Limpopo Province), Rocktail Bay and Umbilo (KwaZulu Natal Province), Irene (Gauteng), Taung and Kgaswane Game Reserve (North West Province), Van der Kloof Dam area (Northern Cape Province) and Maluwa Nature Reserve in Swaziland (Figure 3.1). The collection represented 18 different bat species from 8 families (Table 3.1). Bats were morphologically identified by Dr Teresa Kearney (Ditsong Museum of Natural Science). The necessary permits for collection were obtained.



1: Kruger National Park: N=149

2: Taung: N=2

3: Irene caves: N=18

4: Maluwa nature reserve: N=50

5: Rocktail bay: N=49

6: Umbilo: N=75

7: Kgaswane: N=3

8: Van der Kloof Dam: N=8

Figure 3.1: Map of Southern Africa showing the locations where the bats used in this study was collected from. The dots indicate the location of collection. N: number of samples collected.

Table 3.1: Information about blood samples collected from bats in different locations in South Africa and Swaziland

Family	Bat species	No of samples	Location	Province and country	Date of collection	Laboratory reference no
Pteropodidae	*Epomophorus wahlbergii*	79	Kruger National Park (4)	Limpopo Province: South Africa	2010/02/04	UP 823- 825 & UP 1003
			Umbilo (75)	KwaZulu Natal: South Africa	2007/11/07	UP 209-286
	Rousettus aegyptiacus	7	Kruger National Park (7)	Limpopo Province South Africa	2010/11/10	UP 909-911, UP 933, UP 927-8, UP 711, UP 743-44,
	Epomophorus gambianus	3	Kruger National Park(3)	Limpopo Province South Africa	2010/11/12	UP 1004-1006
Miniopteridae	*Miniopterus natalensis*	26	Irene cave(18)	Gauteng South Africa	2010/04/01	I1-18
			Van der kloof Dam (8)	Northern Cape Province (South Africa)	2010/01/04	UP 810-11, UP 813, UP822-825, UP 829,
Nycteridae	*Nycteris thebaica*	55	Maluwa Nature Reserve (50)	Swaziland	2008/10/11	UP350-400
			Rocktail Bay (5)	KwaZulu Natal: South Africa	2010/04/02	UP 854-6
Vespertillionidae	*Scotophilus spp*	92	Kruger National Park (57)	Limpopo Province: South Africa	2010/02/01	UP 716 & 718, UP 722-23, UP 751, UP 736-742, UP765-767,UP779-812, and UP929-30,UP 954—60, UP 929-30, UP 935
			Rocktail Bay (35)	KwaZulu Natal: South Africa	2010/04/02	UP 845-852, UP 830-834 and UP 865-867UP 1008-1012, UP1014-1022, UP 1034-40
	Nyctecienops schlieffenii	26	Kruger National Park (23)	Limpopo Province: South Africa	2010/02/01	UP 717, UP 719-21, UP 729-30, UP 773-8, UP 813-20
			Rocktail Bay (3)	KwaZulu Natal: South Africa	2010/04/15	Up 827, 846& 868
	Neoromicia nana	24	Kruger National Park (24)	Limpopo Province: South Africa	2010/11/10	UP 931, UP 941-6 & UP 916-26
	Neoromicia capensis	4	Kruger National Park	Limpopo Province South Africa	2010/11/08	UP 915, UP 952

			(2)			
			Kgaswane (2)	North West Province South Africa	2010/12/11	UP 1052-3
	Eptesicus hottentotus	2	Taung (2)	North-West Province: South Africa	2007/06/10	UP 170&173
	Glauconycteris variegata	3	Rocktail Bay (3)	KwaZulu Natal : South Africa	2010/04/02	UP 827,846 & 868
	Pipistrellus rusticus	1	Kruger National Park (1)	Limpopo Province South Africa	2010/02/03	UP 932
Molossidae	*Molosid spp*	13	Kruger National Park (12)	Limpopo Province South Africa	2010/02/03	Up 821-2, UP 757-9 UP 936-8 &UP 965-968
			Kgaswane (1)	North West Province: South Africa	2010/11/12	UP 1007
	Mops condylurus	9	Kruger National Park (9)	Limpopo Province: South Africa	2010/02/02	UP 732-36, UP 750, UP 752, UP770-772
	Chaerephon pumilus	5	Rocktail Bay (5)	KwaZulu Natal: South Africa	2010/04/02	Up 835-839
Rhinolophidae	*Rhinolophus landeri*	1	Kruger National Park(1)	Limpopo Province: South Africa	2010/02/02&2010/02/03	UP 747
Hipposideridae	*Hipposideros caffer*	1	Kruger National Park (1)	Limpopo Province South Africa	2010/11/08&2010/11/11	UP 948
Emballonuridae	*Taphozous mauritianus*	1	Kruger National Park (1)	Limpopo Province South Africa	2010/11/12	UP 953
Total		354				

3.2.2 Total genomic DNA extraction from bat blood samples

Genomic DNA was extracted by using the Qiagen DNeasy blood and tissue kit (Qiagen, Germany) according to the manufacturer's instructions. Briefly, 100 µl of bat blood was resuspended in 100 µl PBS (50mM KH_2PO_4, 150mM NaCl (pH 7.0) (Biowhittaker, USA) and 20 µl Proteinase K (20 mg/ml).The mixture was lysed with 200 µl of buffer AL and incubated at 56℃ for 10 min utes. Two hundred µl of 100% ethanol was added to the lysate. The lysate was transferred to DNeasy mini column and centrifuged at 8000 *x g* for 1 minute. The column was washed twice with 500 µl of buffer AW1 (with 96% ethanol) and AW2 (with 96% ethanol), and centrifuged at 8000 *x g* and 13 400 *x g* for 1 minute and 3 minutes, respectively. DNA was eluted with 100 µl of Buffer AE (10mM Tris-HCl, 0.5 mM EDTA (pH 9.0), followed by incubation for 1 minute and centrifugation at 8000 x g for 1 minute.

3.2.3 Agarose gel electrophoresis

Five µl of the extracted DNA was analysed on a 1% agarose gel stained with ethidium bromide (10mg/µl) (Sigma-Aldrich, USA). A DNA molecular weight marker, 100 bp Plus DNA ladder (Fermentas, USA) was used to determine the molecular size of the products. The gels were resolved at 100 V in an ENURO^{TM} horizontal gel box (Labnet International, USA) and visualized with the use of a UV transilluminator.

3.2.4 Detection of rickettsial DNA in bat blood samples

In this section, the PCR assays developed in section 2.2.1.5, 2.2.1.6, and 2.2.1.8-9 were used to detect rickettsial nucleic acid in the bat blood samples. Briefly, the genomic DNA extracted from 3.2.2 was subjected to a real time PCR targeting the partial fragment of the *gltA* gene of *Rickettsia* as described in section 2.2.1.5. Positive real time PCR samples were further subjected to a conventional and nested PCR targeting the partial *gltA* gene of *Rickettsia* as described in section 2.2.1.6 and 2.2.1.8. The nested PCR was used to increase sensitivity. The *16S rRNA* gene was also amplified as described in section 2.2.1.9 to confirm results obtained from the *gltA* gene analysis The PCR products were analyzed by agarose gel electrophoresis as described in section 3.2.3.

3.2.5 Purification and sequencing of the PCR products

The PCR products were purified by using the Wizard SV Gel and PCR cleanup system (Promega, USA) according to the manufacturer's instructions (as described in section 2.2.1.10). The purified PCR products were sequenced using the ABI Prism BigDye®Terminator Version 3.1 cycle sequencing kit (Applied Biosystems, Germany) (as described section in 2.2.1.11).

3.2.6 Phylogenetic analysis

The sequences were edited and trimmed using the CLC Main Workbench 6 (CLC-bio A/S, Denmark). Pairwise alignment was also performed using the CLC Main Workbench 6 (CLC-bio A/S, Denmark) to determine similarity between the *16S rRNA* and *gltA* gene sequence of *Rickettsia* detected in bats and sequences available in the public domain (Genbank) (Table 3.2 and 3.3). ClustalW was used to create multiple alignments and a maximum parsimony phylogentic tree of the partial *gltA* and *16S rRNA* genes was constructed in Mega 5.05 software using the close-neighbour interchange (CNI) aligorithm (Nei and Kumar, 2000) with complete deletion of missing data and 10 parsimonious tree recovered (Tamura *et al*., 2011). A thousand bootstrap replications were used to estimate nodal reliabitlities of the phylogenetic trees. Bootstrap values higher than 70 and 60 were considered significant, respectively.

Table 3.2: The *gltA* gene sequence of *Rickettsia* species obtained from Genbank and used for maximum parsimony phylogenetic analysis

***Rickettsia* species**	**Genbank accession number**	**Strain**	**Host**	**Geographic origin**	**Reference**
#*R. rickettsii*	NC0098821	Sheila smith	-	-	-
**R. prowazeki*	(NC0009631)	Madrid E	*Homo sapiens* (Human)	France	Anderson *et al.,*1998
$*R. bellii*	(NC007940)	RML-369-L42	▫*Dermacentor variabilis*	France	Roux *et al*., 1996
#*R. massillae*	(NC009900)	MTU5	▫*Rhipicephalus turanicus*	France	Roux *et al.,* 1996
**R. typhi*	NC06142	Wilmington	*Homo sapiens* (Human)	USA	Roux *et al.,* 1996
#*R. Canadensis*	NC009879	Mickel	▫*Haemaphysalis leporispalustris*	Canada	Unpublished
#*R. felis*	NC007109	URRWXcal	*Ctenocephalides felis*	USA	Ogata *et al*., 2005
#*R. japonica*	NC016050	YM	*Homo sapiens* (Human)	Japan	Roux *et al*., 1996
#*R. rickettsii*	NC010263	Iowa	Homo sapiens (Human)	USA	Roux *et al*., 1996
#*R. conorii*	NC003103	Malish 7	Unknown	South Africa	Roux *et al.,* 1996
#*R. peackockii*	NC012730	Rustic	▫*Dermacentor adersonii*	USA	Felsheim *et al*., 2009
# *R. africae*	NC012633	ESF-5	▫*Amblyomma variegatum*	Ethiopia	Roux *et al.,* 1996
$*R. bellii*	(NC009883	0SU-85-389	▫*Dermacentor variabillis*	USA	Roux *et al.,* 1996
#*R. akari*	NC009883	Hartford	*Homo sapiens* (Human)	USA	Roux *et al*., 1996
# Astrakhan fever *Rickettsia*	U59728	A-167	▫*Rhipicephalus pumilo*	Russia	Roux *et al*., 1996
# Astrakhan fever *Rickettsia*	AF10103	Chad 1	▫*Rhipicephalus spp*	Russia	Zhu *et al.,* 2004
#Israeli tick typhus *Rickettsia*	U59727	CDC-1	Homo sapiens (Human)	Israel	Roux *et al.,* 1996
#*R. heiolongjgenisis*	NC051866	N/A	-	Japan	Unpublished
#*R. conorii*	U59730	Seven	*Homo sapiens* (Human)	South Africa	Roux *et al*., 1996
#*R. africae*	U59733	ESF-5	▫*Amblyomma variegatum*	Ethiopia	Roux *et al*., 1996
#*R. rhipicephali*	U59721	3-7-6	▫*Rhipicephalus sanguineas*	USA	Roux *et al*., 1996
# *R. australis*	U59718	Phillips	Homo sapiens (Human)	Australia	Roux *et al*., 1996
#*R. slovaca*	NC016639	13-B	▫*Dermacentor adersoni*	Slovakia	Roux *et al*., 1996
#*R. Helvetica*	EU359285	Isolate 20-2	▫ *Ixodes ricinus*	Switzerland	Borreti *et al*., 2009
#*R.asiatica*	AB297812	JeJu 4	▫*Ixodes pomerantzsvi ex Crocidura*	South Korea	Unpublished
#*R. ammbyolommi*	HM582435	Panama	-	Spain	Bermudez *et al .,* 2011

# indicates spotted fever *Rickettsia*, $ indicates ancestral group *Rickettsia*, * indicates typhus group, ▫ indicates ticks

Table 3.3: The *16S rRNA* gene sequence of *Rickettsia* species obtained from Genbank and used for maximum parsimony phylogenetic analysis

Rickettsia	**Genbank accession number**	**Strain**	**Host**	**Geogarphic orgin**	**Reference**
[&]*R. typhi*	*L36221*	Wilmington	*Homo sapiens* (human)	USA	Roux and Raoult, 1995
[#]*R. slovaca*	L36224	13-B	[¤]*Dermacentor marginalis*	Slovakia	Roux and Raoult, 1995
[#]*R. sibirica*	NR036848	246	[¤]*Dermacentor nuttali*	Siberia	Roux and Raoult, 1995
[#]*R. tarasevechalre*	AM418457	1-337	[¤]I*xodes persulcatus*	Russia	Popov *et al*., 2007
[#]*R. Canadensis*	U51162	2678	[¤]*Haemophysalis leporispalustris*	Canada	Roux and Raoult, 1995
[#]*R. parkeri*	U12461	Maculatum-20	[¤]*Amblyomma maculatum*	USA	Roux and Raoult, 1995
[#]*R. conorii*	U12460	ITT CDC1	*Homo sapiens* (Human)	Israel	Roux and Raoult, 1995
[#]*R. australis*	U12459	Phillips	*Homo sapiens* (Human)	Australia	Roux and Raoult, 1995
[#]*R. akari*	U12458	MK (Kaplan)	*Homo sapiens* (Human)	USA	Roux and Raoult, 1995
[#]*R. rhicephali*	U11019	3-7-6	[¤]*Rhipicephalus sanguineaes*	Spain	Roux and Raoult, 1995
[#]*R. montana*	U11016	ATCC VR 611	[¤]*Dermacentor variabillis*	Ohio	Roux and Raoult, 1995
[#]*R. ambyolomii*	(11012	-	[¤]*Amblyomma americunum*	USA	Roux and Raoult, 1995
[#]*R. rickettsii*	U11021	TT-118	[¤]*Ixodes or Rhipicephalus*	Thailand	Roux and Raoult, 1995
[$]*R. bellii*	U11014	369L42-I	[¤]*Dermacentor variabillis*	USA	Roux and Raoult, 1995
**R. tsutsugamushi*	L362221	Gilliam	*Homo sapiens* (Human)	Japan	Roux and Raoult, 1995
[#]*R. sibrica*	L36218	246	[¤]*Dermacentor nuttalli*	Siberia	Roux and Raoult, 1995
[#]*R. rickettsii*	L366217	R(Bitterroot)	[¤]*Dermacentor andersoni*	Montana	Roux and Raoult, 1995
[#]*R. japonica*	L36213	YM	*Homo sapiens* (Human)	Japan	Roux and Raoult, 1995
[#]*R. massilae*	L36211	Mtu1	*Homo sapiens* (Human)	France	Roux and Raoult, 1995
[#]*R. Helvetica*	L36212	C9P9	[¤]*Ixodes ricinus*	Switzerland	Roux and Raoult, 1995
[#]Astrakhan fever *Rickettsia*	L36100	A167	[¤]*Rhipicephalus pumilo*	India	Roux and Raoult, 1995
[#]*R.conorii*	L36107	Morrocan	Unknown	Morocco	Roux and Raoult, 1995
[#]*R. africae*	L36098	ESF 5	[¤]*Amblyomma variegatum*	Ethiopia	Roux and Raoult, 1995
[#]*R. conorii*	AF541999	Seven	*Homo sapiens* (Human)	South Africa	Roux and Raoult, 1995
Anaplasma mhagocytophylum	JN181064	LT8-3-16	[¤]*Ixodes ricinus*	Lithuinia	Unpublished
Anaplasma marginalae centrale	NC013532	Israel	[¤]*Rhipicephaculs spp.*	Israel	Herndon *et al.,* 2010
Erlichia chaffeensis	NC007799	-	[¤]*Amblyomma americanum*	USA	Dunning-Hotopp *et al.,* 2001
Erlichia canis	NC007354	Jake	[¤]*Rhipicephalus sanguineas*	Algeria	Mavromatis *et al.,* 2006
Wolbachia endosymbiont	NC010981	wPip-nat	*Culex qunquefasciatus* (Mosquito)	USA	Klasson *et al*., 2008

[#] indicates spotted fever *Rickettsia*, [$] indicates ancestral group *Rickettsia*, [*] indicates *Orientia,* [&] indicates typhus group, [¤] indicates ticks

3.2.7 Analysis of the *gltA* and *16S rRNA* gene sequences of *Rickettsia* obtained from bats

For this analysis partial gene (*gltA* and *16S rRNA*) sequences from bats were compared with representative sequences of spotted fever group *Rickettsia* available in the public domain (Table 3.2 and 3.3). The paiwise distance of the partial *gltA* and *16S rRNA* gene datasets was estimated by p-distance method of Mega 5.05 software, with the inclusion of transition and transversion and a paiwise deletion of missing data (Tamura *et al*., 2011).

3.3 Results

3.3.1 Detection of rickettsial cleic acid of in bat blood samples

PCR assays developed in chapter 2 were used and it was possible to generate PCR amplicons for the partial *16S rRNA* and *gltA* genes in 6/354 bats screened. These 6 bats were a *Nycteris thebaica* (UP 368) from Maluwa Nature Reserve (Swaziland), a *Miniopterus natalensis (*I3) from Irene caves (Gauteng), a *Scotophillus dinganii* (UP 847), an *Epomophorus wahlbergi* (UP 826) and a *Glauconycteris variegata* (UP 827) from Rocktail Bay (KwaZulu Natal) and an *Epomophorus wahlbergi* (UP 235) from Umbilo region (KwaZulu Natal). *Rickettsia* DNA concentration was estimated using the standard curve equation. Copy numbers ranged from 613 to 1564 per ng of DNA (Table 3.4).

$$\text{Copy number} = \frac{\text{concentration (g/µl)}}{\text{Length of PCR product x 660}} \times 6.022 \times 10^{23}$$

Table 3.4: The quantification results of *Rickettsia* in southern African bat species

Laboratory ID number	Bat species	Concentration (ng/µl)	Cp values	Copy number per ng of DNA
UP235	*Epomophorus wahlbergi*	50	31.91	650
UP368	*Nycteris thebaica*	55	30.52	716
UP826	*Epomophorus walbergi*	120	27.55	1564
UP827	*Glauconycteris variegata*	93	28.98	1359.3
UP847	*Scotophillus dinganii*	76	28.02	111.01
I3	*Miniopterus natalensis*	42	31.51	613.5

3.3.2 Phylogenetic analysis of the *gltA* and *16S rRNA* sequences of *Rickettsia*

Maximum parsimony phylogenetic trees were constructed comparing the partial *16S rRNA* gene to ensure that the sequences generated were of members within the genus *Rickettsia*. Representative sequences of *Rickettsia* (SFG, TG and AG) and other genera (*Wolbachia*, *Anaplasma* and *Ehrlichia*) were included *(*Table 3.3). The partial *16S rRNA* gene sequences of bats clustered together with members of the genus *Rickettsia* and separately from other genera (Figure 3.2). To attempt to determine the group and species, maximum parsimony phylogenetic trees were constructed comparing the partial *gltA* gene sequences and all the representative groups were included (Table 3.2). The partial *gltA* gene sequences obtained from bat samples clustered together with the SFG *Rickettsia* and was separate from other groups (Figure 3.3).

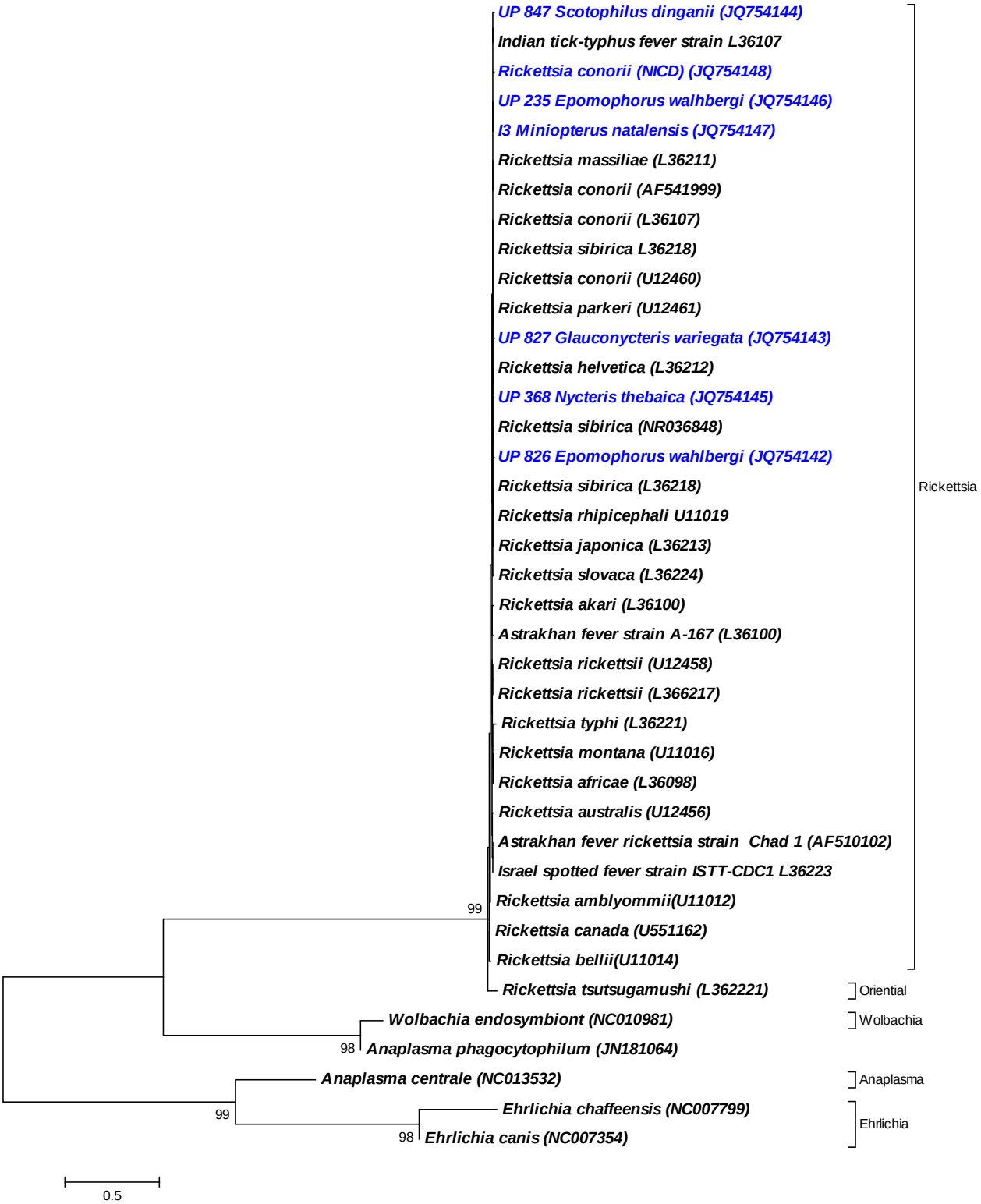


Figure 3.2: Maximum parsimony phylogenetic analysis of the partial *16S rRNA* of *Rickettsia*. Representative sequences of *Rickettsia* from other hosts were included. Sequences obtained from this study are indicated in blue and are represented by the laboratory number, bat species and accession number. Only bootstrap values higher than 70% are shown in the figure.

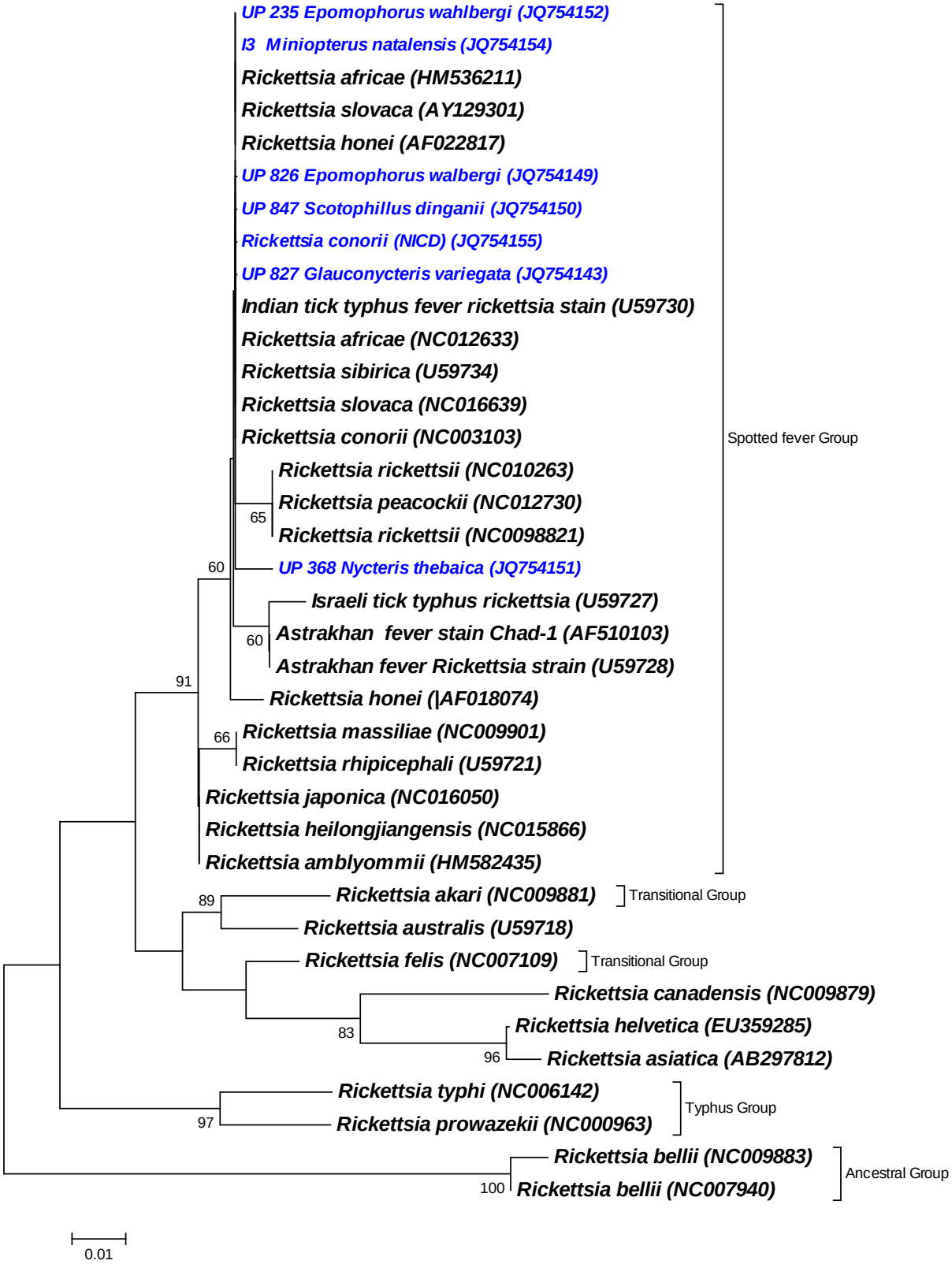


Figure 3.3: Maximum parsimony phylogenetic analysis of the partial *gltA* gene of *Rickettsia*. Representative sequences of *Rickettsia* from other hosts were included. Sequences obtained from this study are indicated in blue and are represented by the laboratory number, bat species and their accession number. Only bootstrap values higher than 70% are shown in the figure.

3.3.3 Analysis of the *gltA* and *16S rRNA* gene sequences of *Rickettsia* detected in bats

Pairwise distance estimation was performed for the partial *16S rRNA* gene and *gltA* gene using the nucleotide sequences. Based on sequences comparisons, the results showed that the bat sequences were closely related to each other (DNA similarity (99-100%) and other spotted fever group *Rickettsia* with DNA similarity of 98.0 to 100% with the partial *gltA* (Figure 3.4) and *16S rRNA* genes (Figure 3.5), respectively.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	*R. conorii (NC003103)*																						
2	*R. rickettsii (NC010263)*	0.007																					
3	*R. peacockii (NC012730)*	0.007	0.000																				
4	*R. massiliae (NC009901)*	0.014	0.021	0.021																			
5	*R. slovaca (NC016639)*	0.000	0.007	0.007	0.014																		
6	*R. sibirica (U59734)*	0.000	0.007	0.007	0.014	0.000																	
7	*R. rickettsii (NC0098821)*	0.007	0.000	0.000	0.021	0.007	0.007																
8	*R. rhipicephali (U59721)*	0.014	0.021	0.021	0.000	0.014	0.014	0.021															
9	*R. japonica (NC016050)*	0.007	0.014	0.014	0.007	0.007	0.007	0.014	0.007														
10	*R. heilongjiangensis (NC015866)*	0.007	0.014	0.014	0.007	0.007	0.007	0.014	0.007	0.000													
11	*R. africae (NC012633)*	0.000	0.007	0.007	0.014	0.000	0.000	0.007	0.014	0.007	0.007												
12	*Astrakhan fever strain Chad-1 (AF510103).*	0.007	0.014	0.014	0.021	0.007	0.007	0.014	0.021	0.014	0.014	0.007											
13	*Indian tick typhus fever Rickettsia (U59730)*	0.000	0.007	0.007	0.014	0.000	0.000	0.007	0.014	0.007	0.007	0.000	0.007										
14	*Israeli tick typhus Rickettsia (U59727)*	0.014	0.021	0.021	0.028	0.014	0.014	0.021	0.028	0.021	0.021	0.014	0.007	0.014									
15	*Astrakhan fever Rickettsia strain (U59728)*	0.007	0.014	0.014	0.021	0.007	0.007	0.014	0.021	0.014	0.014	0.007	0.000	0.007	0.007								
16	*UP 827 G. variegata (JQ754153)*	0.000	0.007	0.007	0.014	0.000	0.000	0.007	0.014	0.007	0.007	0.000	0.007	0.000	0.014	0.007							
17	*R. conorii (NICD) (JQ754155)*	0.000	0.007	0.007	0.014	0.000	0.000	0.007	0.014	0.007	0.007	0.000	0.007	0.000	0.014	0.007	0.000						
18	*UP 368 N. thebaica (JQ754151)*	0.007	0.014	0.014	0.021	0.007	0.007	0.014	0.021	0.014	0.014	0.007	0.014	0.007	0.021	0.014	0.007	0.007					
19	*UP 847 S. dinganii (JQ754150)*	0.000	0.007	0.007	0.014	0.000	0.000	0.007	0.014	0.007	0.007	0.000	0.007	0.000	0.014	0.007	0.000	0.000	0.007				
20	*UP 826 E. walhbergi (JQ754149)*	0.000	0.007	0.007	0.014	0.000	0.000	0.007	0.014	0.007	0.007	0.000	0.007	0.000	0.014	0.007	0.000	0.000	0.007	0.000			
21	*I2 M. natalensis (JQ754154)*	0.000	0.007	0.007	0.014	0.000	0.000	0.007	0.014	0.007	0.007	0.000	0.007	0.000	0.014	0.007	0.000	0.000	0.007	0.000	0.000		
22	*UP 235 E. walhbergii (JQ754152)*	0.000	0.007	0.007	0.014	0.000	0.000	0.007	0.014	0.007	0.007	0.000	0.007	0.000	0.014	0.007	0.000	0.000	0.007	0.000	0.000	0.000	

Figure 3.4 Pairwise distance matrix analysis of the partial *gltA* gene (nucleotides) of *Rickettsia*. Representative *Rickettsia* sequences from other animal hosts were included. Sequences obtained from this study are indicated in blue and are represented by the laboratory number, bat species and accession number.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	*R. slovaca (L36224)*																							
2	*R. sibirica (NR036848)*	0.003																						
3	*R. parkeri (U12461)*	0.016	0.014																					
4	*R. sibirica (L36218)*	0.003	0.000	0.014																				
5	*R. conorii (U12460)*	0.003	0.000	0.014	0.000																			
6	*R. rickettsii (U12458)*	0.005	0.003	0.016	0.003	0.003																		
7	*R. amblyommii (U11012)*	0.011	0.008	0.019	0.008	0.008	0.011																	
8	*R. sibirica (L36218)*	0.003	0.000	0.014	0.000	0.000	0.003	0.008																
9	*R. rhipicephali(U11019)*	0.003	0.000	0.014	0.000	0.000	0.003	0.008	0.000															
10	*R. rickettsii (L366217)*	0.005	0.003	0.016	0.003	0.003	0.000	0.011	0.003	0.003														
11	*R. conorii (NICD) (JQ754147)*	0.003	0.000	0.014	0.000	0.000	0.003	0.008	0.000	0.000	0.003													
12	*UP 235 E. walhbergi(JQ754146)*	0.003	0.000	0.014	0.000	0.000	0.003	0.008	0.000	0.000	0.003	0.000												
13	*UP 827 G. variegata((JQ754143)*	0.003	0.000	0.014	0.000	0.000	0.003	0.008	0.000	0.000	0.003	0.000	0.000											
14	*UP 847 S. dinganii ((JQ754144)*	0.003	0.000	0.014	0.000	0.000	0.003	0.008	0.000	0.000	0.003	0.000	0.000	0.000										
15	*UP 826 E.wahlbergi ((JQ754142)*	0.003	0.000	0.014	0.000	0.000	0.003	0.008	0.000	0.000	0.003	0.000	0.000	0.000	0.000									
16	*UP 368 N. thebailca (JQ754145)*	0.003	0.000	0.014	0.000	0.000	0.003	0.008	0.000	0.000	0.003	0.000	0.000	0.000	0.000	0.000								
17	*I3 M. natalensis (JQ754146)*	0.003	0.000	0.014	0.000	0.000	0.003	0.008	0.000	0.000	0.003	0.000	0.000	0.000	0.000	0.000	0.000							
18	*R. africae (L36098)*	0.005	0.003	0.016	0.003	0.003	0.005	0.011	0.003	0.003	0.005	0.003	0.003	0.003	0.003	0.003	0.003	0.003						
19	*Astrakhan fever Rickettsia strain Chad (AF510102)*	0.005	0.003	0.016	0.003	0.003	0.005	0.008	0.003	0.003	0.005	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.005					
20	*Mediterranean spotted fever (AF541999)*	0.003	0.000	0.014	0.000	0.000	0.003	0.008	0.000	0.000	0.003	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.003				
21	*Indian tick-typhus fever strain (L36107)*	0.003	0.000	0.014	0.000	0.000	0.003	0.008	0.000	0.000	0.003	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.003	0.000			
22	*Israel spotted fever strain ISTT-CDC 1(L36223)*	0.005	0.003	0.016	0.003	0.003	0.005	0.008	0.003	0.003	0.005	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.005	0.000	0.003	0.003		
23	*Astrakhan fever Rickettsia (L36100)*	0.005	0.003	0.016	0.003	0.003	0.005	0.011	0.003	0.003	0.005	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.005	0.005	0.003	0.003	0.005	

Figure 3.5 Pairwise distance matrix analysis of the partial *16S rRNA* gene (nucleotide) of *Rickettsia*. Representative *Rickettsia* sequences from other animal hosts were included. Sequences obtained from this study are indicated in blue and are represented by the laboratory number, bat species and accession number.

3.4 Discussion

Previous studies on *Rickettsia* in bats have reported only levels of seropositivity (Reeves *et al*., 2006a; D Àuria *et al*., 2010). In this study, the aim was to attempt to detect *Rickettsia* nucleic acid in bats from southern African using specific PCR assays optimized in chapter 2. This study provides the first report on the detection of *Rickettsia* DNA from bat blood samples. A total of six samples (from a panel of 354) from South African and Swaziland bat species were found to be PCR positive. These samples were from five different bat species – viz. *Epomophorus wahlbergi*, *Nycteris thebaica, Glauconycteris variegata, Scotophillus dinganii* and *Miniopterus natalensis*.

Comparison of the *gltA* and *16S rRNA* gene sequence generated in this study with sequences available in the public domain indicated that the sequences generated from bats were closely related to other *Rickettsia s*pecies known to occur in this region. To confirm these results, maximum parsimony phylogenetic analysis comparing the partial *16S rRNA* gene sequences was conducted. Analysis of the partial *16S rRNA* gene sequence revealed that *Rickettsia* identified in this study were closely related to members under the genus *Rickettsia* and were distantly related to other genera within the order *Rickettsiales*. Analysis of this gene did not distinguish between *Rickettsia* groups or species. This was expected because the *16S rRNA* gene is highly conserved in the genus *Rickettsia* and it is only suitable for identification of *Rickettsia* to the genus and sometimes to the group level (Roux and Raoult, 1995; Fournier *et al.,* 2003; Fournier and Raoult, 2009). Since a partial *16S rRNA* was amplified in our study, analysis of that region could only phylogenetically classify to the genus level.

In order to attempt to identify the group, species and strain of bat derived rickettsia sequences, maximum parsimony phylogenetic analysis were performed using the partial *gltA* gene. All the sequences clustered together with the spotted fever group (SFG) and were separated from the typhus (TG), transitional (TRG) and ancestral group (AG). This was supported by a strong bootstrap value (>70%). The *gltA* gene analysis did not reveal the species and strain of the sequences. Although, the *gltA* gene has been reported to identify *Rickettsia* to the species level (Fournier *et al.,* 2003; Fournier and Rauolt, 2009), in this study sequences could only be generated from a nested PCR product (200bp). Thus, analysis of this small fragment did not provide a strong bootstrap support and as a result bat isolates were only identified to

the group level. In future, studies on other genes such as the *ompA* gene might provide a better discrimination among these *Rickettsias*. The *ompA* gene is variable and the 5-end region particularly useful for identification of closely related *Rickettsia* species, for example in the spotted fever group (La-Scola and Raoult, 1997; Roux and Raoult, 2000 Fournier and Raoult, 2009). Currently, there is no single gene that can identify *Rickettsia* to the strain level (Fournier *et al.,* 2007; Founier and Raoult, 2009). However, a newly developed technique such as multi spacer typing has proved to be useful. This technique makes use of non-coding regions to define *Rickettsia* to the strain level because they are less subject to evolutionary pressure when compared to coding regions (Fournier *et al.,* 2007). This technique might be useful in differentiating *Rickettsia* species detected in this study. The advent of high throughput sequencing has allowed rapid sequencing of several genomes of *Rickettsia* (Sentausa *et al*., 2012; Fournier *et al.,* 2012). This has allowed several genomes of *Rickettsia* to be compared and studied in detail. Future studies could involve the use of high throughput sequencing to determine the similarities and differences between *Rickettsia* genes detected in bats with genes from known rickettsial species from other hosts.

Based on nucleotide aligments, the sequences of the *gltA* and *16S rRNA* of bats were highly similar (DNA similarities 99-100%). The level of sequence similarity to other spotted fever group rickettsias, ranged from 98%-100% and 99.7-100%, respectively. This was in agreement with previous findings indicating the percentages of sequence similarity among *Rickettsia* species from the same group ranging from 99.8%-100% and 99.7-100% for the *16S rRNA* and *gltA* gene sequences, respectively (Roux and Rauolt, 1995; Fournier *et al.,* 2003: Zhu *et al*., 2004; Fournier and Raoult, 2009).

Bats roost in natural (caves, rocks, tree branches bird nest etc) and in man-made structures (buildings, mines and bridges). Their occupations in man-made structures could bring closer associations with humans and their livestock (Wong *et al*., 2007). In this study bats that were positive for rickettsial DNA were captured from different locations ranging from caves (*Miniopterus natalensis*), grasslands savannahs (*Epomophorus wahlbergi*), and buildings (*Glauconycteris variegata*) and over a swimming pool (*Scotophillus dinganii*). The close association of some of these bats to humans could present a potential health risk (Reeves *et al.,* 2006a; Pearce and O'shea, 2007). Loftis *et al.,* (2005), reported a natural rickettsial infection in a bat tick

Carios kelleyii. These ticks infest on *Eptesicus fuscus* which inhabit on building roofs and bridges, closer to humans. Subsequent studies by Reeves *et al.,* (2006a) showed that these ticks can transmit rickettsial pathogens to susceptible hosts during a blood meal. Gill *et al*., (2004) showed that these ticks can feed on humans in the absence of their primary host, bats. Although transmission of *Rickettsia* to humans by bat ectoparasites has never been investigated, these findings from previous studies suggest a potential transmission.

In Africa, human rickettsial infections are reported annually (Parola *et al.,* 2005; Frean and Blumberg, 2007, Henderson and Sexton, 2009). In some cases, there is an emergence of rickettsial infections in areas where it has never been reported (Pretorius and Birtles, 2002; Pretorius *et al*., 2004; Socolovschi e*t al*., 2010; Richards *et al.,* 2010). Surveillance for these pathogens in new hosts, such as bats, might shed light in revealing new possible vectors/reservoirs for some *Rickettsia* species and possibly lead to the discovery of new *Rickettsia* species.

Chapter 4: Detection of *Bartonella* DNA in southern African bat species

4.1 Introduction

Members of the genus *Bartonella* are highly fastidious, facultative intracellular bacteria that are transmitted between mammalian hosts by blood feeding arthropods such as fleas, biting flies, lice and ticks (Andersonn and Neumann, 1997; Breitschwertd *et al.,* 2010; Chomel and Kasten, 2010). *Bartonella* are highly adaptive organisms that have the ability to invade the host immune system and cause persistent bacteremia by occupying the host erythrocytes (Breitschwerdt *et al.,* 2010). At least five *Bartonella* species have been implicated in human and animal diseases in Africa (Breitswercht *et al.,* 2010). These species are *B. bovis* (Raoult *et al*., 2005) , *B. henselae* (Kelly *et al*., 1998; Frean *et al*., 2002; Molia *et al.,* 2004; Trataris*,* 2011*), B. quintana* (Raoult *et al*., 1998; Trataris *et a*l., 2011), *B. vinsonii subsp berkoffii* (Kerniff *et al*., 2010) and *B. clarriadgeiae* (Gundi *et al*.,2004). However, the most common *Bartonella* species in South Africa is *B. henselae*, an agent of cat scratch disease (CSD) (Frean *et al*., 2002; Trataris, 2011), and it is common in immunocompromised individuals (HIV-infected individuals) (Frean *et al.,* 2002; Trataris, 2011) cats, dogs, lions and cheetahs (Molia *et al*., 2004; Kelly *et al.,* 2004; Gumbi et al., 2004; Pretorius *et al*., 2004b).

Bartenollosis in bats have been poorly investigated. To our knowledge, there have been only four publications that have reported *Bartonella* infections in bats. In the first study in Cornwall, United Kingdom, *Bartonella* DNA was detected in 5/60 bats that were screened (Concannon *et al.,* 2005). Although, *Bartonella* isolates were not cultured in this study, detection of bacterial DNA indicated a possible infection. Kosoy *et al.,* (2010) in Kenya, reported *Bartonella* infection in 30.2 % (106/331) of the bats tested and 58 unique *gltA Bartonella* genotypes were identified. Another study in Guatemala, Bai *et al*., (2011) reported *Bartonella* infection in 33% (39/119) of the bats and 21 unique *Bartonella gltA* genotypes were identified. A recent study by Lin *et al*., (2011) in Taiwan reported a possible novel *Bartonella* strain in *Miniopterus schreibersii*. In all these studies, the mode of transmission of *Bartonella* to bats is unknown but it was hypothesized that ectoparasites may be involved in the transmission of *Bartonella* species between bats (Kosoy *et al*., 2010; Bai *et al.,* 2011; Lin *et al.,* 2011). A recent study in western Africa reported *Bartonella* species in bat-flies, *Cyclopodia greefi greefi* (Diptera: Nycteribliidae) that were removed from *Eidolon helvum*. In this study, a *Bartonella* sequences detected from bat flies were

similar to sequences of *Bartonella* species that were previously isolated in *Eidolon helvum* bat from Kenya, indicating that ectoparasites may responsible for transmission of *Bartonella* species between bats (Billeter *et al.,* 2012)

The aim of this chapter was to identify and characterize *Bartonella* species that may circulate in southern African bat species. Bat blood samples were collected from different locations in South Africa and Swaziland. Genomic DNA was extracted and nucleic acid detection methods evaluated in chapter 2 were used to amplify the *gltA* gene of *Bartonella*. The *gltA* gene of *Bartonella* detected in bats was sequenced and phylogenetic relationships were determined. Here we report the detection of *Bartonella* DNA for the first time in southern African bat species.

4.2 Materials and methods

4.2.1 Bat blood samples

The blood samples used in Chapter 3 (Figure 3.1 and and Table 3.1) were analyzed.

4.2.2 Total genomic DNA extraction

The extracted DNA material that was used for detection of rickettsial nucleic acid in bat blood samples was further analyzed for *Bartonella* DNA. The total genomic DNA was extracted by using the DNeasy blood and tissue kit (Qiagen, Germany) according to the manufacturer's instructions (as described in section 3.2.2).

4.2.3 Detection of *Bartonella* nucleic acid in bat blood samples

A partial fragment of the *gltA* gene (379 bp) of *Bartonella* was amplified as described in section 2.4.4.5

4.2.4 Agarose gel electrophoresis

Once amplification was complete, 5 µl of the PCR products were analyzed on a 2% agarose gel stained with ethidium bromide (10 µg/ml) (Sigma-Aldrich, USA). The gels were resolved at 100V in an ENURO^{TM} horizontal gel box (Labnet International, USA). A DNA molecular weight marker, 100 bp Plus DNA ladder (Fermentas, USA) was used to determine the size of the products and visualized under a UV light.

4.2.5 Purification and sequencing of the PCR products

The PCR products were purified using the Wizard SV gel and PCR cleanup system (Promega, USA), according to the manufacturer's instructions (as described in section 2.2.1.10). Purified products were sequenced by using the ABI Prism®

BigDye® Terminator Version 3.1 cycle sequencing kit (Applied Biosystems, Germany) (as described in section 2.2.1.11).

4.2.6 Phylogenetic analysis

The sequences were edited and trimmed using the CLC Main Workbench 6 (CLC-bio A/S, Denmark). Pairwise alignment was performed by using the CLC Main Workbench 6 (CLC-bio A/S, Denmark) to determine similarity between the *gltA* sequences of *Bartonella* detected in bats and other *gltA* sequence of *Bartonella* available in the public domain (Genbank:NCBI). Representative sequences of *Bartonella* available in the public domain were included (Table 4.1). *Bartonella* sequences obtained from Kenyan bats were included in the analysis. ClustalW was used to create multiple alignments. Neighbour joining trees are Kimura-2 parameter model distance with transversion and transition ratio of 2:1 (Tamura *et al*., 2011). Bootstrap analyses were made with 1000 replications. Bootstrap values higher than 70 were considered significant.

Table 4.1: The *gltA* gene sequences of *Bartonella* species that were used for neighbour joining phylogenetic analysis

***Bartonella* species**	**Genbank accession number**	**Host**	**Strain**	**Geographical origin**	**Reference**
B. vinsonii	U28074	-	-	USA	Unpublished
B. birtlesii	AF204272	*Apodemus spp* (Wood mouse)	ISB 325	France	Bermond *et al.,* 2000
B. alsiatica	AF204273	*Oryctolagus cuniculus* (Rabits)	ISB 382	France	Bermond *et al.,* 2000
B. elizabethae	U28072	-	-	USA	Unpublished
B. tribocorum	AJ005494	*Rattus norvegicus* (Rats)	ISB 506	France	Heller *et al*., 1998
B. grahammi	Z70016	*Homo sapiens* (Human)	V2	Peru	Birtles and Raoult,1997
B. henselae	L378987	-	Houstan-1	-	Alsmark *et al*., 2004
B. koehlerae	AF176091	*Felis catus* (Cats)	C-29	USA	Droz *et al.,* 1999
B. taylorii	AF191502	-	Wm9	-	Birtles, 1999
B. coopersplainsesis	EU111803	*Rattus tunneyi* (Pale field rats)	AUST/NH2	Australia	Gundi *et al*., 2009
B. phoeensis	AY515126	*Rattus norvegicus* (Rats)	16120	France	Gundi *et al.,* 2004
B. clarriadgeiae	U84386	-	Houstan-2	USA	Clarridge *et al.,* 1995
B. rochalimeae	DQ683195	*Homo sapiens* (Human)	BMGH	Peru	Eremeeva *et al*., 2007
B. bovis	AF293394	*Capreolus capreolus* (Roe deer)	91-4	France	Bermond *et al*., 2002
B. bacilliformis	U28076	-	Kc584	USA	Unpublished
B. doshiae	AF207827	*Rattus norvegicus* (Rodents)	Wbs011	United Kingdom	Birtles., 1999
B. tamaie	DQ395177	*Homo sapiens* (Human)	Th239	Thailand	Kosoy *et al.,* 2008
B. australis	DQ538395	*Macropus giganteus* (Kangaroos)	NHL	Australia	Fournier *et al.,* 2006

4.2.7 Analysis of the *gltA* gene sequences of *Bartonella* obtained from bats

For this analysis, the partial *gltA* gene sequences derived from bats were compared with representative sequences of *Bartonella* from Africa (Table 4.2). Bat sequences from Kenya were also included in this analysis. The distance estimation of the *gltA* gene dataset was performed by the p-distance method of Mega 5.05software with the inclusion and transversion and the pairwise deletion of missing data (Tamura *et al.,* 2011).

Table 4.2: The *gltA* gene sequences obtained from GenBank used for pairwise distance analysis

***Bartonella* spp**	**Genbank accession number**	**Strain**	**Host**	**Country**	**Reference**
B. elizabethae	JN523214	Sm6145V1	Suncus murinus (Asian mask shrew)	Viet-Nam	Unpublished
B. elizabethae	FJ9468491	KK5	*Canis familiaris* (Dog)	Thailand	Bai *et al*., 2009
B.elizabethae	GU056193	BR03	Ectoparasites	-	Unpublished
B.elizabethae	Z00009	F9251	-	-	Birtles and Raoult,1997
B.elizabethae	GQ225710	45-00311	*Homo sapiens* (Human)	Thailand	Kosoy, 2009
B. henselae	NC005956	Houstin -1	*Homo sapiens* (Human)	USA	Norman *et al*., 1995
B. quintana	NC005955	Touluse	Louse/Human	USA	Birtles and Raoult, 1997
B. clarridgeiae	NC014932	Strain 73	Ectoparasites	USA	Birtles and Raoult, 1997

4.3 Results

4.3.1 Detection of *Bartonella* DNA in southern African bats

The PCR assays developed in chapter 2 were used and it was possible to generate PCR amplicons for the partial *gltA* gene in 13/354 samples that were were analyzed. The bat species were *Miniopterus natalensis* (UP 810, 811, 825, 824 and 829) from Van der Kloof dam area (Northern Cape), an *Epomophorus wahlbergi* (UP 1003) from Kruger National Park (Pafuri: Limpopo province), *Miniopterus natalensis* (I2, I4 and I6) from Irene (Gauteng) and *Nycteris thebaica* (UP 351, 361, 369 and 384) from Maluwa Nature Reserve (Swaziland).

4.3.2 Phylogentic analysis

The *gltA* gene (310 bp) fragments of the *Bartonella* genotypes from bats were sequenced and phylogenetically analyzed together with representative sequences from Kenyan bats. All the *Bartonella* genotypes detected in this study, except a genotype from Limpopo (UP 1003), clustered together and were closely related to *Bartonella grahamii* (Cluster II-V) (Figure 4.1). UP 1003 from *E. wahlbergi* clustered together with *B. elizabethae* in a separate monophyletic cluster (cluster I) and this was supported by a strong bootstrap value (>90%) (Figure 4.1). Unlike the previous studies on *Bartonella* in bats (Kosoy *et al*., 2010; Bai *et al*., 2011; Lin *et al*., 2011),

Bartonella genotypes detected in our study were closely related to previously described *Bartonella* species from rodents.

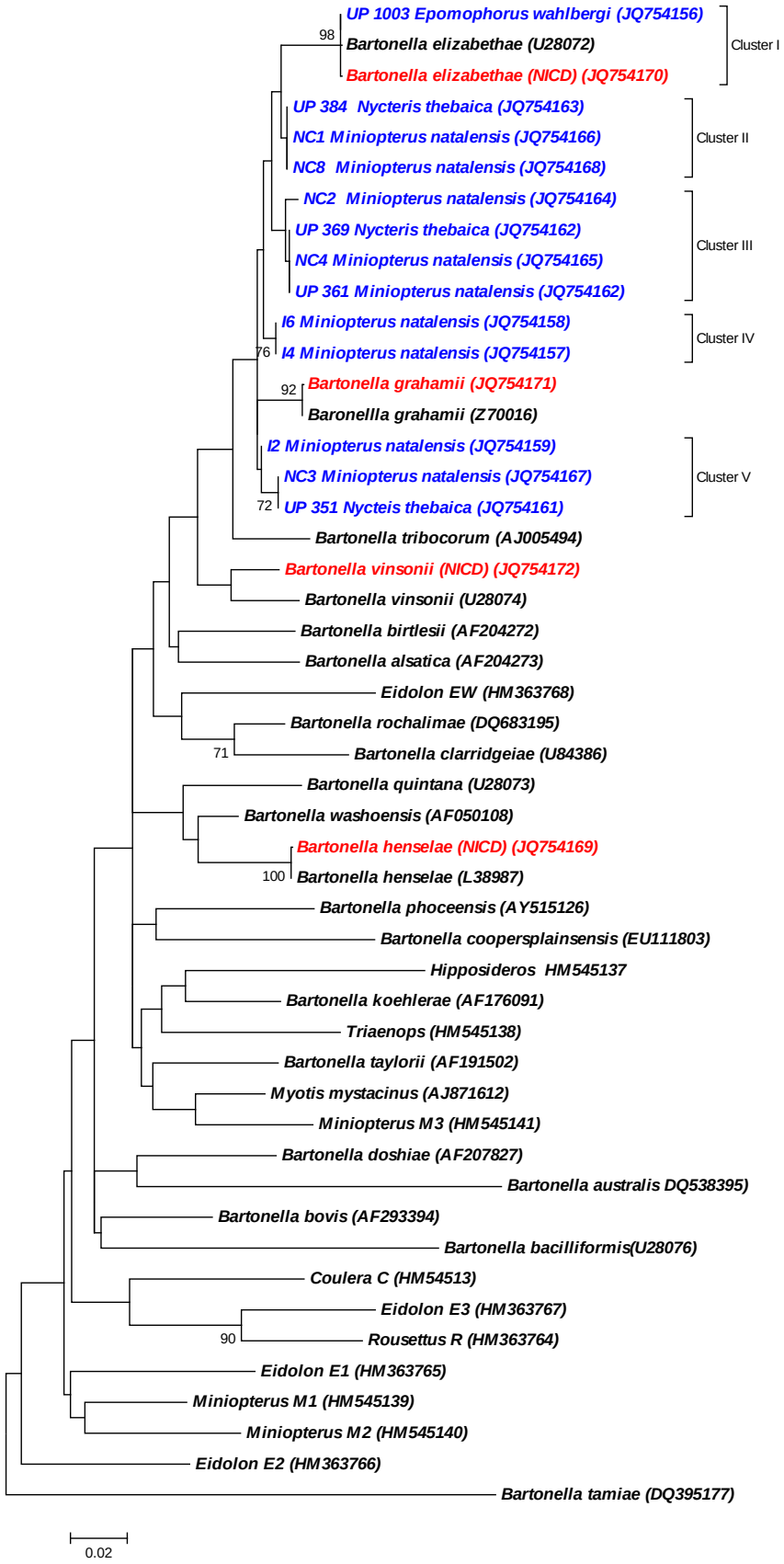


Figure 4.1: Neighbour joining phylogenetic analysis of the partial *gltA* gene of *Bartonella.* Representative sequences obtained from *Bartonella* associated with bats as well as with other hosts, were included. Sequences obtained from this study are represented by the laboratory number, bat species and accession number and indicated in blue. Sequences indicated in red are from positive controls. Only bootstrap values higher than 70% are shown in the figure.

4.3.3 Sequence analysis of the *gltA* gene of *Bartonella* detected in bats

All the *Bartonella* sequences generated from bats in this study were similar to each other (DNA similarity of 94-100%) and were closely related to *B. grahamii* (Figure 4.1 and 4.2), the only exception was the sequence from UP 1003 (*E. wahlbergii*) which was closely related to other *B. elizabethae* isolates, with DNA similarity of 95-100% (Figure 4.1 4.2 4.3 and 4.4). To ensure that this was not a contamination of positive control, pairwise alignment was performed using Bioedit v.7 software (Hall, 1999). The sequence obtained from *E. wahlbergi* was 95 % similar to *B. elizabethae* (positive control) (Figure 4.3 and 4.4) and *N. thebaica* and *M. natalensis* derived sequences were 94-98% similar to *B. grahamii* (positive control) (Figure 4.5). The bat derived sequences were distantly related to *Bartonella* genotypes from Kenyan bats (79-94% similar) (Figure 4.1 and 4.2).

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
1	*B. henselae* (L38987)																													
2	*UP 1003 (JQ754156)	0.15																												
3	ᵝUP 351 (JQ754161)	0.16	0.08																											
4	#I6 (JQ754158)	0.09	0.05	0.07																										
5	#UP 825 (JQ754164	0.13	0.07	0.03	0.06																									
6	#UP 811 (JQ754165	0.09	0.05	0.07	0.02	0.04																								
7	#UP 824 (JQ754166)	0.12	0.03	0.05	0.02	0.04	0.02																							
8	#Up 810 (JQ754167)	0.10	0.04	0.06	0.01	0.05	0.01	0.01																						
9	#I2 (JQ754159)	0.10	0.04	0.06	0.01	0.05	0.01	0.01	0.00																					
10	#UP 829 (JQ754168)	0.12	0.03	0.05	0.02	0.04	0.02	0.00	0.01	0.01																				
11	ᵅ *Eidolon* EW (HM363768)	0.06	0.14	0.15	0.12	0.11	0.09	0.10	0.10	0.10	0.10																			
12	ᵅ *Miniopterus* M3 (HM545141)	0.11	0.13	0.16	0.08	0.15	0.10	0.10	0.09	0.09	0.10	0.14																		
13	ᵅ *Myotis mystacinus* (AJ871612)	0.09	0.07	0.11	0.04	0.10	0.06	0.06	0.05	0.05	0.06	0.13	0.06																	
14	ᵅ *Eidolon* E3 (HM363767)	0.13	0.20	0.20	0.14	0.19	0.16	0.16	0.15	0.15	0.16	0.16	0.16	0.14																
15	ᵅ Roesettus R (HM363764)	0.14	0.18	0.21	0.16	0.20	0.16	0.16	0.15	0.15	0.16	0.13	0.15	0.14	0.08															
16	ᵅ *Coulera* C (HM545136)	0.15	0.16	0.21	0.15	0.17	0.15	0.15	0.16	0.16	0.15	0.15	0.18	0.15	0.15	0.18														
17	ᵅ *Hipposideros* (HM545137)	0.12	0.15	0.16	0.12	0.13	0.09	0.12	0.11	0.11	0.12	0.12	0.19	0.14	0.22	0.21	0.16													
18	ᵅ Trianops (HM545138)	0.14	0.12	0.15	0.12	0.14	0.12	0.11	0.10	0.10	0.11	0.12	0.16	0.10	0.15	0.14	0.16	0.14												
19	ᵅ *Miniopterus* M1 (HM545139)	0.17	0.14	0.17	0.10	0.16	0.13	0.13	0.12	0.12	0.13	0.18	0.15	0.15	0.18	0.16	0.18	0.22	0.19											
20	ᵅ *Miniopterus* M1 (HM545140)	0.16	0.10	0.14	0.09	0.13	0.09	0.09	0.08	0.08	0.09	0.14	0.13	0.12	0.16	0.10	0.22	0.15	0.13	0.09										
21	ᵅ *Eidolon* E1 (HM363765)	0.21	0.16	0.21	0.18	0.20	0.18	0.18	0.16	0.16	0.18	0.19	0.21	0.19	0.18	0.14	0.22	0.25	0.16	0.12	0.13									
22	ᵅ *Eidolon* E2 (HM363766)	0.17	0.19	0.19	0.18	0.20	0.18	0.17	0.16	0.16	0.17	0.18	0.18	0.18	0.14	0.10	0.17	0.20	0.16	0.14	0.15	0.14								
23	#UP 361(JQ754162)	0.09	0.05	0.07	0.00	0.06	0.02	0.02	0.01	0.01	0.02	0.12	0.08	0.04	0.14	0.16	0.15	0.12	0.12	0.10	0.09	0.18	0.18							
24	ᵝUP 384 (JQ754163)	0.09	0.05	0.07	0.00	0.06	0.02	0.02	0.01	0.01	0.02	0.12	0.08	0.04	0.14	0.16	0.15	0.12	0.12	0.10	0.09	0.18	0.18	0.00						
25	ᵝUP 369 (JQ754162)	0.09	0.05	0.07	0.00	0.06	0.02	0.02	0.01	0.01	0.02	0.12	0.08	0.04	0.14	0.16	0.15	0.12	0.12	0.10	0.09	0.18	0.18	0.00	0.00					
26	#I4 (JQ754157)	0.10	0.04	0.06	0.01	0.05	0.01	0.01	0.00	0.00	0.01	0.10	0.09	0.05	0.15	0.15	0.16	0.11	0.10	0.12	0.08	0.16	0.16	0.01	0.01	0.01				
27	*B. grahamii* (Z70016)	0.14	0.05	0.03	0.04	0.06	0.04	0.02	0.03	0.03	0.02	0.13	0.13	0.08	0.19	0.19	0.18	0.14	0.13	0.15	0.12	0.20	0.17	0.04	0.04	0.04	0.03			
28	*B. elizabethae* (U28072)	0.17	0.03	0.05	0.08	0.04	0.08	0.06	0.07	0.07	0.06	0.16	0.16	0.10	0.21	0.20	0.20	0.18	0.14	0.16	0.13	0.17	0.20	0.08	0.08	0.08	0.07	0.08		
29	*B. clarridgeiae* (U84386)	0.12	0.12	0.15	0.10	0.14	0.13	0.11	0.12	0.12	0.11	0.10	0.15	0.13	0.11	0.14	0.13	0.19	0.10	0.15	0.14	0.19	0.16	0.10	0.10	0.10	0.12	0.13	0.14	
30	*B. vinsonii* (U28074)	0.10	0.11	0.07	0.06	0.06	0.08	0.08	0.07	0.07	0.08	0.15	0.13	0.09	0.16	0.17	0.20	0.15	0.15	0.16	0.15	0.18	0.18	0.06	0.06	0.06	0.07	0.10	0.08	0.14

Figure 4.2**:** Pairwise distance matrix analysis of the partial *gltA* gene of *Bartonella.* Sequences obtained from this study are indicated in blue and are represented by the laboratory number and accession number and bat species. * indicates- *Epomophorus wahlbergi*, # indicates *Miniopterus natalensis,* ᵝ indicates *Nycteris thebaica* and ᵅ indicates bats from Kenya.

		1	2	3	4	5	6	7
1	*B. elizabethae* (JF523414)							
2	B. elizabethae (GQ225710)	0.05						
3	*B. elizabethae* (GU056193)	0.00	0.05					
4	*B. elizabethae* (FJ946849)	0.00	0.05	0.00				
5	*B. elizabethae* (Z70009)	0.00	0.05	0.00	0.00			
6	UP 1003 *E. wahlbergi* (JQ754156)	0.05	0.05	0.05	0.05	0.05		
7	*B. elizabethae* (NICD)(JQ754170)	0.00	0.05	0.00	0.00	0.00	0.05	

Figure 4.3: Pairwise distance matrix analysis of the partial *gltA* gene of *Bartonella elizabethae* and UP1003. The sequence obtained from this study (blue) is represented by the laboratory number, bat species and accession number. The positive control is indicated in red

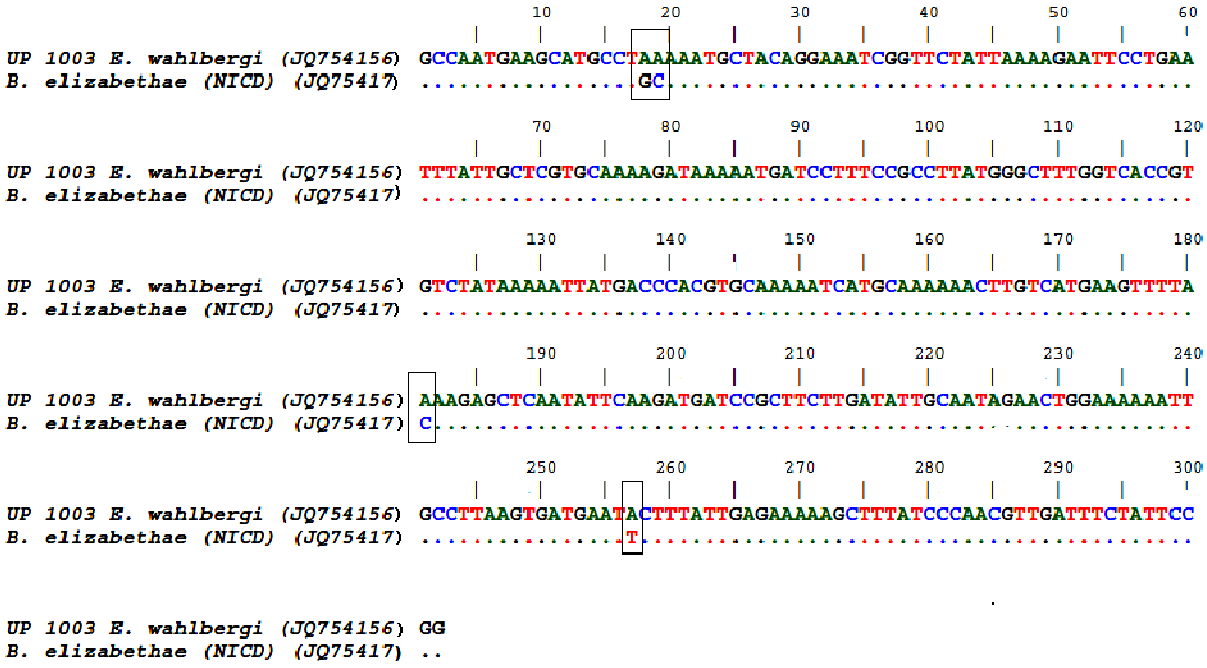


Figure 4.4: Pairwise analysis of the partial *gltA* gene sequence generated from the positive control and *Bartonella elizabethae* sequence obtained from the bat blood sample (UP1003). The sequence obtained from this study is represented by the laboratory number, bat species and accession number.

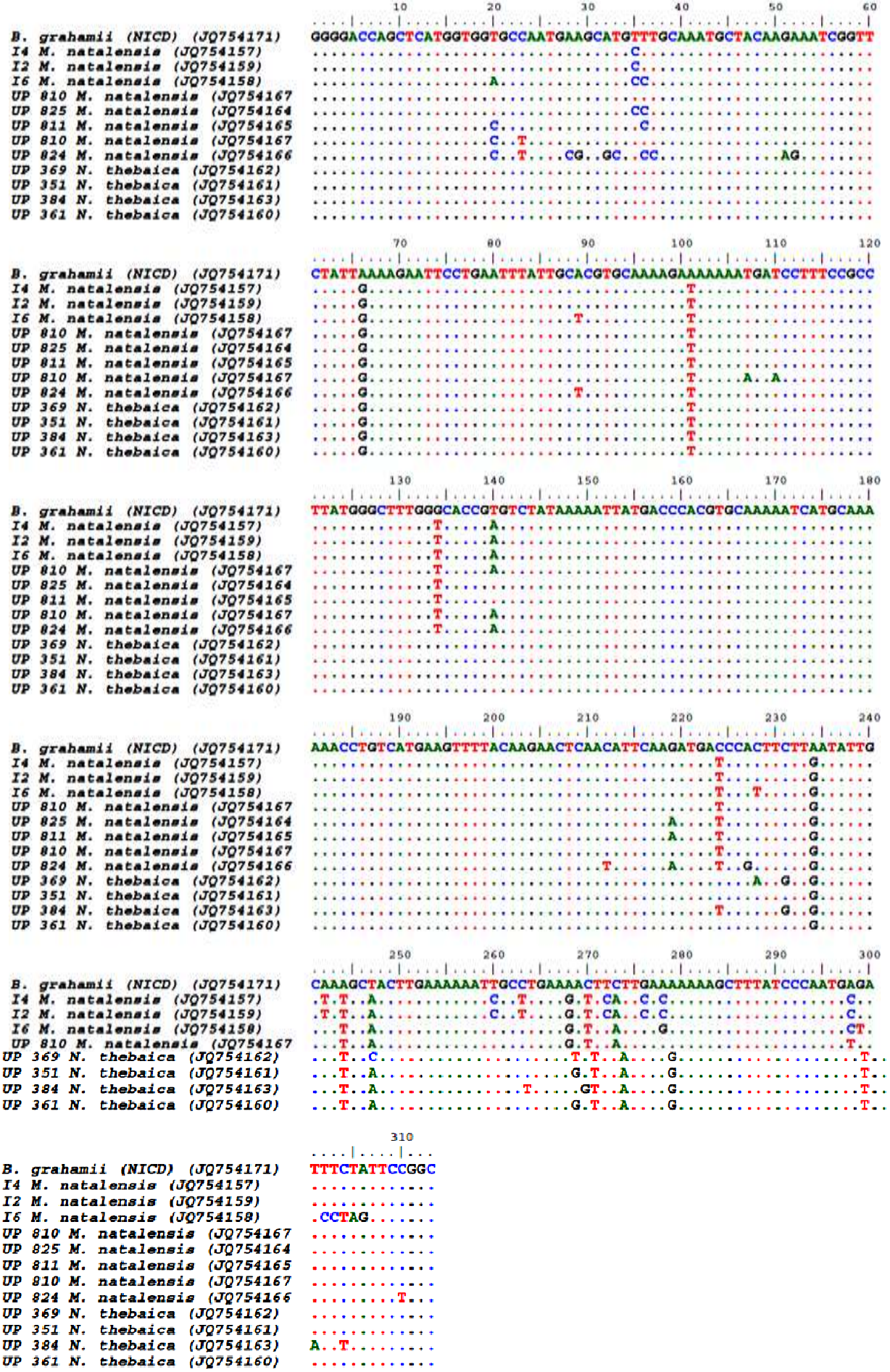


Figure 4.5: Pairwise analysis of the partial *gltA* gene sequence generated from the positive control, *B. grahamii* and sequences obtained from the bat blood samples. The sequences obtained from this study are represented by the laboratory number, bat species and accession number.

4.4. Discussion

The incidence of *Bartonella* infections in southern African bat species was investigated using PCR and we report for first time, the detection of *Bartonella* DNA in South African and Swaziland bat species. *Bartonella* DNA was detected in 13/354 bats that were screened and these were five *Miniopterus natalensis* from Van der Kloof dam area (Northern Cape), an *Epomophorus wahlbergii* from Kruger National Park (Limpopo), three *Miniopterus natalensis* from Irene (Gauteng) and four *Nycteris thebaica* from Maluwa Nature Reserve (Swaziland). Although we did not attempt to isolate *Bartonella* species in culture as in previous studies in Kenya (Kosoy *et al.,* 2010), Guatemala (Bai *et al*., 2011) and Taiwan (Lin *et al*., 2011), detection significant amount of bacterial nucleic acid indicates that these bats could be infected. The source of infection was unknown but arthropods seem to be a good candidate (Breitschwerdt and Kordick, 2000; Breitschwerdt *et al*., 2010).

Among the 18 bat species that were screened for *Bartonella* DNA, only 3 species (*Miniopterus natalensis*, *Epomophorus wahlbergii* and *Nycteris thebaica*) tested positive. Unlike the other studies in Kenya and Guatemala where specific locations were targeted for surveillance, in our study bats were randomly collected from different areas. These areas included culverts under the road (*M. natalensis* from Northern Cape and *N. thebaica* from Swaziland), caves (*M. natalensis* from Gauteng), and a under bark of trees (*E. walhbergi*). In this study *M. natalensis* showed to be the bat species that is at high risk of infection irrespective of site of collection as also demonstrated in chapter 3 for *Rickettsia* infection. *Miniopterus natalensis* is insectivorous and usually roosts in caves and can reach high population density. They can migrate up to 260 km between warmer maternity caves (Monadjem *et al*., 2010). The large colony size of these bats together with social behaviors such as mutual grooming, biting during courtship and mating, might facilitate the transmission of *Bartonella* between these bats either through direct contact or by arthropod vectors. *Nycteris thebaica*, like *M. natalensis* is insectivourous and can be found in smaller colonies in their roosts (Monadjem, 1998). *Bartonella* pathogens can be transmitted between bats that roost together. *Epomophorus wahlbergi* on the other hand frugivorous and either roost singly or in small groups in urban areas (Monadjem *et al*., 2010). Kosoy *et al*. (2010) reported novel *Bartonella* species in straw coloured fruit bat, *Eidolon helvum*. Subsequent

studies by Billeter *et al*., (2012), reported *Bartonella* infections in bat flies removed from these bats. Therefore, it can be hypothesised that transmission of *Bartonella* to *E. walhbergi* occurred through arthropod vectors because there is limited information available to suggest any alternative mechanism for vector transmission.

Phylogenetic analysis showed that *gltA Bartonella* sequences obtained from *M. natalensis* and *N. thebaica* were closely related to *Bartonella grahamii* and *E. walhbergi* derived *Bartonella* sequence was closely related to *B. elizabethae*. This is the first study to report these findings. *Bartonella grahamii* is the most prevalent *Bartonella* species in wild rodents (Birtles *et al.,* 1995; Hofmeister *et al.,* 1998; Holmberg *et al*., 2003; Jardine *et al*., 2005; Telfer *et al.,* 2007; Inoue *et al.,* 2008) and has been implicated with neuroretinitis in humans (Kerkhoff *et al*., 1999). *Bartonella elizabethae* is also rodent-borne (Ellis *et al*., 1999; Pretorius *et al.,* 2004c) and has been isolated from dogs (Mexas *et al.,* 2002; Kerniff *et al*., 2010) and humans (Daly *et al*., 2003).

These findings were not similar to what was observed in the study in Kenya, Guatemala and Taiwan (Kosoy *et al*., 2010; Bai *et al.,* 2011, Lin *et al*., 2011). In Kosoy *et al.,* (2010), the genotypes detected from bats were “host specific” and were distantly related to previously described *Bartonella* strains from other animal hosts. In Bai *et al*., (2011), “host specificity” was not observed; however, they found that a single/individual bat was infected with multiple *Bartonella* species. Like, Kosoy *et al*., (2010), the genotypes detected in this study were distantly related to known *Bartonella* species from other animal hosts. In Taiwan, Lin *et al.,* (2010) reported that genotypes detected in bats were closely related to *Bartonella* strains detected in stray dogs. Moreover, two unique genotypes were identified in *Miniopterus* spp. were distantly related to bat genotypes from the same area and known *Bartonella* species from other animal hosts.

Analysis of the *gltA* gene again showed that our sequences were distantly related to sequences derived from Kenyan bats. Unlike previous studies of *Bartonella* in bats, our study did not reveal “host specificity”, but it reports that *Bartonella* sequences detected in *Miniopterus natalensis* and *Nycteris thebaica* are closely related. This finding raises questions especially since these bats were collected from different geographical locations. In South Africa, Monadjem *et al*., (2010) reported that

Miniopterus natalensis can migrate up to 260 km between warmer maternity caves, where females give birth in summer (eg De Hoop Guano cave in the Western cape or other caves in Limpopo or Gauteng) (Monadjem *et al*., 2010). *Nycteris thebaica* on the other hand does not migrate; instead they overwinter in warm conditions. Monadjem *et al*., (2010) again reported the presence of *N. thebaica* in De Hoop Guano cave in the Western Cape during winter and autumn. These species overwinter in this cave just beyond maternity dome of *Miniopterus natalensis.*

Bartonella genotypes detected in our study could potentially represent new rodent-borne strains; however, studies on other genes are needed to describe novel strains. The *gltA* gene is highly conserved, it cannot discriminate closely related *Bartonella* strains (Agan *et al.,* 2002; La-Scola *et al*., 2003; Zeaiter *et al*., 2003). Therefore, future studies on other genes such as *ribC* and *rpoB* might provide a better discrimination between these *Bartonellas*. These genes have a higher diversity when compared to the *gltA* gene (Zeaiter *et al.,* 2003). The Ad-Hoc committee for the re-evaluation of the species definition in bacteriology emphasizes that for an organism to be considered a new species/strain, at least a minimum of five genes including protein coding gene should be characterized (Stackebrand *et al*., 2002; Fournier *et al.,* 2003; Fournier and Raoult, 2009).

Future studies should also involve surveillance of these pathogens in bat ectoparasites. With studies reporting novel *Bartonella* species/strains in bats (Lin *et al*., 2011), there is a need to further investigate if bats could be reservoir hosts for new species.

Chapter 5: Concluding remarks

This study has contributed to a small number of studies dedicated to the identification of pathogens in southern African bat species and has shown that these bats harbor the bacterial pathogens; *Rickettsia* and *Bartonella.* Although, *Bartonella* and *Rickettsia* species were not isolated in culture, the detection of pathogen-specific nucleic acid in 1.7% and 3.7% of a cohort of 354 bats, indicated infection with *Rickettsia* and *Bartonella* respectively. Generally, ectoparasite infestation is known as a common mode of *Rickettsia* and *Bartonella* transmission and these pathogens are transmitted to susceptible hosts by blood-sucking arthropods (Dick *et al.,* 2006; Reeves *et al.,* 2006a; Pearce and O'Shea, 2007; Breitschwerdt *et al.,* 2010; Chomel and Kasten, 2010). Since bats carry a wide range of ectoparasites, the same epidemiological and transmission mechanisms are likely to apply in the case of *Rickettsia* and *Bartonella* infections in bats.

The first step in this study was to evaluate PCR assays for the detection of *Rickettsia* and *Bartonella* DNA. A real time, nested and conventional PCR targeting the partial *gltA* and *16S rRNA* genes were optimized for the detection of *Rickettsia* in bats. Among all the assays that were developed, the real time and nested PCR showed the highest detection limit as also reported in previous studies (Stenos *et al.,* 2005; Wölfel *et al.,* 2008). These techniques proved to be useful in detecting bacterial DNA *(Rickettsia*) even when present in low concentrations. For detection of *Bartonella*, the conventional PCR targeting the partial *gltA* gene was developed. This PCR was also able to detect *Bartonella* DNA in bat blood samples.

The citrate synthase gene *(gltA*) and the *16S rRNA* gene were useful in detecting rickettsial DNA in the bat blood samples, but these genes were not good candidate genes for identification of *Rickettsia* to species and strain level. Thus, the species dectected in this study were only identified phylogenetically to genus and group level. These findings were in agreement with findings from previous studies (Roux and Raoult, 1995; Fournier *et al*., 2003; Fournier and Raoult, 2009). It was concluded that analysis of other genes such as the *ompA* gene will provide better variation and could enable the identification of *Rickettsia* to the species, because this gene has a higher diversity when compared to the *16S rRNA* and the *gltA* genes (Roux and Raoult,2000; Fournier and Raoult, 2009). The use of recently developed techniques such as multi spacer typing might be useful in differentiating closely

related *Rickettsia* strains detected in this study (Fournier *et al*., 2007). Future studies could involve techniques such as high throughput sequencing, since they allow rapid sequencing of multiple bacterial genes.

The citrate synthase (*gltA*) proved to be a good candidate gene for detection of *Bartonella* in bat blood samples. *Bartonella* sequences detected in bats were closely related to each other and other rodent-borne *Bartonella* species but were distantly related to *Batonella* species derived from Kenyan bats. Analysis of this gene did not allow proper identification to the strain level. It was concluded that analysis of other genes such as *ribC*, *groEL* and *rpoB* might offer a better discrimination between these *Bartonellas*.

Several bat species in Africa have been reported positive for bacterial and viral pathogens (Kosoy *et al*., 2010; Markotter *et al*., 2006; Kuzmin *et al*., 2010a). Because of lack of proper surveillance, these pathogens are not frequently reported. The first step to understand the role of bats in public and veterinary health is to determine the incidence of pathogens. This was our main aim in this study. Future work will involve isolation of the bacteria and further characterization using multiple genes. Although, isolation of *Bartonella* and *Rickettsia* are an ideal method to indicate an active infection, these methods have major drawbacks. *Rickettsia* and *Bartonella* are slow growing, highly fastidious bacteria and isolation in culture is difficult and requires specialized environments such as BSL 3 laboratories (Agan *et al*., 2002; Wölfel *et al.,* 2008). Therefore, in our study we only used nucleic acid detection methods because they are highly sensitive and can detect bacterial DNA even when present in low concentration in the sample.

To date bat associated *Bartonella* and *Rickettsia* infection has never been reported to humans. Studies have shown that climate changes and development of infrastructure has led destruction of the natural habitats of bats and this has brought a closer association of bats with humans (Wong *et al*., 2007). In Africa, human infections caused by some members of *Bartonella* and *Rickettsia* infections are reported annually (Frean *et al.,* 2008; Trataris, 2011). In some cases there is an emergence of rickettsial or bartonella infections in areas where it was considered endemic (Socolovschi, *et al*., 2010; Mediannikov *et al*., 2010). Thus, surveilllance for

these pathogens in new host such as bats might help us identify new reservoir hosts/vectors and possibly identify new species of *Rickettsia* and *Bartonella*.

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