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Rickettsial Diversity and Human Rickettsiosis in South Africa

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Rickettsial Diversity and Human Rickettsiosis in South Africa

By

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SUMMARY

Rickettsial Diversity and Human Rickettsiosis in South Africa

by

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Rickettsia are a diverse group of organisms said to fit in somewhere between viruses and bacteria. They are found globally and cause a plethora of diseases in humans, animals and occasionally in arthropod hosts. Rickettsial pathogenicity is variable with a broad spectrum of associated maladies with severity ranging from mild symptoms to a high rate of case fatality. Clinicians are increasingly having to manage patients presenting with diseases caused by one of a wide range of rickettsias incidentally infecting humans bitten by an arthropod vector. Clinicians are encouraged to maintain awareness of the spectrum of diseases caused by *Rickettsia* species to better recognize and treat afflicted patients. If an arthropod reservoir can opportunistically bite

a human, it can transmit its harbored rickettsial species onto the human. In order to better understand the rickettsial diversity and human rickettsiosis in South Africa, a two-part study was conducted: the first part investigated diagnostic aspects including commercial kit comparability and inter-laboratory comparison (ILC); and the second part examined the rickettsial species involved in severe cases of tick bite fever (TBF) in South Africa and the harborage of *Rickettsia* species by several tick species collected at various locations. The findings are presented herein.

The various serological kits available in South Africa were compared in Chapter 3 to determine whether the various kits would produce consistent results for a panel of specimens. Significant differences between all the kits were observed, thus the null hypothesis was rejected. The deficiencies of commercial diagnostic offerings were highlighted, including the necessity for in-house evaluation, validation and adaptation as required e.g. setting assay cut-off values for an endemic population. In Chapter 4, an inter-laboratory assessment results were reported. The ILC was aimed at gauging performance and agreement of serological TBF results across a standardised clinical specimen panel by several participating laboratories. Although the ILC was a once-off “snap shot” assessment, it was highlighted that South African laboratories would benefit from routine ILC programs, for both serological and molecular-based techniques, to improve proficiency and assess technique reliability. Additionally, passive surveillance programs for known *Rickettsia* ‘hot spot’ areas would provide data necessary to facilitate test optimization criteria e.g. cutoff values and baseline titers.

A case series of patients presenting with suspected Crimean-Congo haemorrhagic fever (CCHF) but diagnosed with TBF in South Africa, between 2011-2018 was described in Chapter 5. Clinical aspects and genetic characterization of the implicated *Rickettsia* species were presented and it was highlighted that TBF is an important differential diagnosis for CCHF, especially since geographic distribution, primary vectors and symptom presentations overlap for the two diseases. Clinicians are encouraged to submit eschar swabs and/or eschar biopsies, for polymerase chain

reaction (PCR) analysis in addition to blood samples submitted, to improve the likelihood of detecting *Rickettsia*. Finally, in Chapter 6, *Rhipicephalus* ticks were found to be harboring two recently-identified South African *Rickettsia* species: ‘Candidatus’ *R. muridii* and ‘Candidatus’ *R. rhabdomydis*, and *H. rufipes* ticks demonstrated a larger *Rickettsia* species diversity with ticks found to be carrying *R. sibirica* subsp. *mongolitimonae*, and possibly two to three currently uncharacterized species.

RESEARCH OUTPUTS

Trataris-Rebisz, A.N., Rossouw, J., Markotter, W., Frean, J.A. and Weyer, J., 2019. Spotted fever rickettsiosis in South Africa: evaluation of laboratory diagnostic capacity and inter-laboratory comparison of serological testing. *South African Medical Journal*, 109(4), pp.223-226.

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DECLARATION

I hereby declare that the dissertation, which I hereby submit for the degree Philosophiae Doctor (Microbiology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

ETHICS STATEMENT

The author, whose name appears on the title page of this dissertation, has obtained, for the research described in this work, the applicable research ethics approval.

The author declares that she has observed the ethical standards required in terms of the University of Pretoria's Code of Ethics for Researchers and the Policy guidelines for responsible research.

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ABREVIATIONS

ADP	adenosine di-phosphate
ATP	adenosine tri-phosphate
AFI	acute febrile illness
AG	ancestral group
ALT	alanine transaminase
ATBF	African tick bite fever
ATCC	American Type Culture Collection
ANI	average nucleotide identity
AST	aspartate transaminase
BSC	biological safety cabinet
BSL-3	biosafety level 3
CCHF	Crimean-Congo haemorrhagic fever
CEZPD	Centre for Emerging Zoonotic and Parasitic Diseases
CF	complement fixation
CO	cut-off control
COI	cytochrome c oxidase subunit I
CRP	C-reactive protein
CRT	calreticulin
DALRRD	Department of Agriculture, Land Reform and Rural Development
DDT	di-chloro-di-phenyl-tri-chloro-ethane
DDH	DNA-DNA hybridization
df	degrees of freedom
DFA	direct fluorescent antibody test
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EC	Eastern Cape Province
ELISA	enzyme-Linked Immunosorbent Assay

F	female
FS	Free State Province
G-C	guanine-cytosine
gltA	citrate-synthase gene
GP	Gauteng Province
H	high
H3	nuclear histone 3
HEL	human erythroleukemia
ICNP	International Code of Nomenclature of Prokaryotes
ICU	intensive care units
IFA	immunofluorescence assay
IgG	immunoglobulin G
IgM	immunoglobulin M
ILC	inter-laboratory comparison
ITS-2	internal transcribed spacer 2
IV	intravenous
KNP	Kruger National Park
L	low
LAR	lymphangitis-associated rickettsiosis
LPS	lipopolysaccharide
LPSN	List of Prokaryotic names with Standing in Nomenclature
M	male
MCL	maximum composite likelihood
mtDNA	mitochondrial DNA
MEGA7	Molecular Evolutionary Genetics Analysis version 7.0
MgCl ₂	magnesium chloride
MIF	micro-Immunofluorescence
MLST	multilocus sequence typing
MP	Maximum Parsimony
MS	misspelling

MSF	Mediterranean Spotted Fever
Mwm	molecular weight marker
N	no
NC	negative control
NCP	Northern Cape Province
NICD	National Institute for Communicable Diseases
N OBS	none observed
NVP	not validly published
NW	North West Province
O.D.	optical density
Omp	outer membrane protein
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PPE	personal protective equipment
RC	reactive control
PFGE	pulsed-field electrophoresis genetic analyses
RFLP	restriction fragment length polymorphism
RG3	risk group 3
RMSF	Rocky Mountain Spotted Fever
rRNA	ribosomal ribonucleic acid
SA	South Africa
SANAS	South African National Accreditation System
SCA	surface cell antigen
SDW	sterile distilled water
SFG	spotted fever group
SPR	Subtree-Pruning-Regrafting
TBD	tick-borne diseases
TBE	tris/borate/EDTA
TBF	tick bite fever
TBP	tick-borne pathogens

TG	typhus group
TOT	transovarial transmission
TRG	transitional group
UKN	unknown
VBZD	vector-borne zoonotic diseases
VHF	viral haemorrhagic fever
VP	validly published
v/v	volume per volume
WB	western blotting
WBC	white blood cell
WC	Western Cape Province
WF	Weil-Felix
WGS	whole-genome sequencing
w/v	weight per volume
Y	yes

CHAPTER 1 – INTRODUCTION

1.1 BACKGROUND

Historically, '*Rickettsia*' has been the generic term used to describe small and fastidious bacteria for which no other classification is available (Merhej and Raoult, 2011). This has, over the years, changed with the enhancement of molecular techniques (Fournier and Raoult, 2009). Rickettsias now are recognized as arthropod-vectorized pathogens responsible for a range of emerging infectious diseases known as Rickettsioses (Fournier and Raoult, 2009). There are currently four (4) groups of diseases commonly identified as rickettsioses:

- (i) diseases caused by Rickettsias including both the spotted fever group (SFG) and the typhus group (TG),
- (ii) scrub typhus due to *Orientia tsutsugamushi* (formerly *Rickettsia tsutsugamushi*),
- (iii) human ehrlichioses and anaplasmosis caused by the family Anaplasmataceae, and
- (iv) Q fever due caused by *Coxiella burnetii*, which was recently removed from the family *Rickettsiales* (Parola et al., 2008, Walker et al., 2003).

Currently, there are six (6) genera in the order *Rickettsiales*, namely *Rickettsia*, *Neorickettsia*, '*Candidatus Aquarickettsia*', *Diplorickettsia*, '*Candidatus Phycorickettsia*', and *Piscirickettsia*. There are 143 named *Rickettsia* species, with at least 20 recognized as human pathogens (Table 1.1) (Parola et al., 2013, Raoult and Maurin, 2008, Shpynov et al., 2018). Ticks are reportedly the foremost reservoirs and key vectors of many *Rickettsia* species, having been detected in a myriad of tick genera, including *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Rhiphicephalus* and *Ixodes* (Azad and Beard, 1998). Ticks are, however, not the only vectors associated with *Rickettsia* transmission with many rickettsias associated with lice, fleas (particularly cat fleas, *Ctenocephalides felis*), mites (Fournier and Raoult, 2009, Raoult and Maurin, 2008) and even mosquitoes (*Anopheles gambiae* and *Aedes albopictus*) (Berrang-Ford et al., 2012). It has been suggested that every rickettsial species has potential for human pathogenesis if its arthropod reservoir is capable of biting humans (La Scola and Raoult, 1997). The scope and importance of recognized tick-associated rickettsial pathogens has increased

considerably in the past two and a half decades. A number of tick-borne rickettsial species previously considered non-pathogenic, are now being linked with human infections, whilst new *Rickettsia* species of undetermined pathogenicity are continuously being detected in or isolated from ticks around the world (Parola et al., 2013). The molecular examination and detection of *Rickettsia* species carried in various South African tick vectors is reported in Chapter 6.

The global public health impact is presumed to be reasonably high despite the true impact, through loss of productivity, being largely unknown (Mahajan, 2012). In humans, some *Rickettsias* are the causative agents of diseases including epidemic typhus and various spotted fevers (Fournier et al., 1998b, Parola and Raoult, 2006, Perlman et al., 2006, Shaw et al., 2001, Hawley et al., 2007). The most common manifestations of rickettsial infections include fever and headache. Inoculation eschars are sometimes associated with cutaneous eruptions and vasculitis, caused by organism propagation in the endothelial lining of capillaries, and indicate disease pathogenesis (Raoult and Roux, 1997, Cowan, 2000).

Rickettsial illness is usually diagnosed by serological testing, however antibodies are generally only observed in the second week of illness. A specific diagnosis is often unavailable until after the patient has recovered or died (La Scola, and Raoult 1997). There are various commercially available diagnostic assays available, including Weil-Felix, immunofluorescence assay (IFA), latex agglutination, indirect haemagglutination, immunoperoxidase assay, complement fixation (CF) test, enzyme-linked immunosorbent assay (ELISA) (Blanda et al., 2017), latex agglutination, western immunoblot, micro-immunofluorescence (MIF) and line probe assay (Sood et al., 2013). Commercially-available serological diagnostic assays are examined in Chapter 3 of this project thesis. Chapter 4 is a published chapter (Trataris-Rebisz et al., 2019) that reports on the status of serological diagnostic testing for *Rickettsia* in South African laboratories.

The occurrence of typhus parallels the history of war and famine from its first description in the 16th century (Fuxelius et al., 2007, Mahajan, 2012). Disease outbreaks followed armies moving up through Europe from the Mediterranean region resulting in soldier fatalities spreading into the civilian populations (Fuxelius et al., 2007, Fang et al., 2017). Typhus has historically emaciated

armies, prisons, and refugee camps, and helped shape war outcomes (Fang et al., 2017). Hygiene measures were implemented during the first and second World Wars in an attempt to control the spread of the human body louse, but millions of European soldiers were nevertheless infected with epidemic typhus (Fuxelius et al., 2007). In Soviet Union and Eastern Europe, an astounding 30 million cases occurred during the first world war with an estimated three million deaths (Mahajan, 2012). During the second world war, scrub typhus caused more deaths among South-East Asia Allied forces than the fighting per se (Fuxelius et al., 2007). The course of history has been altered by epidemic typhus by annihilating armies and civilian populations during times of war (Fang et al., 2017, Valbuena and Walker, 2009). Typhus is not the only *Rickettsia* that manifests infections with high-consequence outcomes. The apparent effectiveness of *Rickettsia* as select agents has been cause for concern for many years. There has been much debate about whether they could be used as biological weapons, two species in particular, *Rickettsia prowazekii* (the agent of epidemic typhus) and *Rickettsia rickettsii* (the agent of Rocky Mountain spotted fever (RMSF)) are considered select agents on the bioterrorism watch list (Fuxelius et al., 2007). There are at least eleven (11) species that are considered risk group 3 (RG3) pathogens, namely; *Rickettsia africae*, *Rickettsia akari*, *Rickettsia australis*, *Rickettsia conorii*, *Rickettsia heilongjiangensis*, *Rickettsia japonica*, *Rickettsia prowazekii*, *Rickettsia rickettsia*, *Rickettsia sibirica*, *Rickettsia tsutsugamushi* and *Rickettsia typhi* (Table 1.1). These RG3 pathogens are considered high-consequence pathogens with dual use potential whereby organisms can be manipulated for nefarious purposes, and are therefore subject to strict regulation globally.

Table 1.1. Current list of validated *Rickettsia* species with published nomenclature (<https://psn.dsmz.de/search?word=rickettsia> - accessed: 10/07/2023)

#	<i>Rickettsia</i> species	Published validity	Group classification	Transmission vector	First isolation / vector species	16S rRNA gene accession number	Type strain	Risk group classification	Reference
1	<i>Rickettsia aeschlimannii</i>	VP	SFG	Tick	<i>Hyalomma marginatum</i>	U74757	Mc16	2	(Beati et al., 1997)
2	<i>Rickettsia africae</i>	VP	SFG	Tick	<i>Amblyomma hebraeum</i>	L36098.1	Z9-Hu	3	(Kelly et al., 1996)
3	' <i>Candidatus Rickettsia africanseptentrionalis</i> '	MS	SFG (TRG)	Tick	<i>Argas dewae</i> <i>Ornithodoros occidentalis</i> , <i>Ornithodoros erraticus</i> and <i>Ornithodoros normandi</i>	MN905457- MN905458	no culture isolated	UKN	(Buysse and Duron, 2020)
	' <i>Candidatus Rickettsia africanseptentrionalis</i> '	NVP							
4	' <i>Candidatus Rickettsia africaustralis</i> '	NVP		Tick	<i>Unspecified</i>	None available	no culture isolated	UKN	(Essbauer et al., 2018)
5	<i>Rickettsia akari</i>	VP	SFG (TRG)	Mite	<i>Allodermanyssus sanguineus</i>	L36099	ATCC VR-148; MK	3	(Huebner et al., 1947)
6	' <i>Candidatus Rickettsia amblyommatis</i> '	NVP	SFG	Tick	<i>Amblyomma americanum</i>	U11012.1	CRIRC RAM004; CSUR- P2882; WB- 8-2	2	(Labruna et al., 2004)
	<i>Rickettsia amblyommatis</i>	VP							(Karpathy et al., 2016)
	' <i>Candidatus Rickettsia amblyommii</i> '	MS							(Labruna et al., 2004)
7	' <i>Candidatus Rickettsia andeana</i> '	NVP	SFG	Tick	<i>Amblyomma maculatum</i> and <i>Ixodes boliviensis</i>	None available	no culture isolated	UKN	(Jiang et al., 2005)
	' <i>Candidatus Rickettsia andeanae</i> '	MS							
8	' <i>Candidatus Rickettsia angusta</i> '	NVP	SFG (AG)	Tick	<i>Ixodes angustus</i>	None available	no culture isolated	UKN	(Anstead and Chilton, 2013)
	' <i>Candidatus Rickettsia angustus</i> '	MS							
9	' <i>Candidatus Rickettsia anophelessinensis</i> '	MS	SFG	Mosquito	<i>Anopheles sinensis</i>	None available	no culture isolated	UKN	(Guo et al., 2016)
	' <i>Candidatus Rickettsia anophelis</i> '	NVP							

10	<i>'Candidatus Rickettsia antechini'</i>	NVP	SFG	Tick	<i>Ixodes antechini</i>	None available	no culture isolated	UKN	(Owen et al., 2006)
11	<i>'Candidatus Rickettsia asemboensis'</i>	MS	SFG	Flea	<i>Ctenocephalides felis</i>	JWSW01000015	ATCC VR-1827; CDC CRIRC RAS001; DSM 100172; NMRCii	UKN	(Jiang et al., 2013)
	<i>'Candidatus Rickettsia asembonensis'</i>	NVP							(Maina et al., 2016)
	<i>Rickettsia asembonensis</i>	VP							
12	<i>Rickettsia asiatica</i>	VP	SFG	Tick	<i>Ixodes ovatus</i>	AF394906	ATCC VR-1593; CSUR R2; IO-1	1	(Fujita et al., 2006)
13	<i>Rickettsia australis</i>	VP	SFG (TRG)	Tick	<i>Ixodes holocyclus and Ixodes tasmani</i>	U12459	NIAID Phillips 32; Phillips	3	(Philip, 1950)
14	<i>'Rickettsia azadi'</i>	NVP	SFG (TRG)	Flea	<i>Ctenocephalides felis</i>	None available	ELB	2	(Radulovic et al., 1995)
15	<i>'Candidatus Rickettsia barbariae'</i>	MS	SFG	Tick	<i>Rhipicephalus turanicus</i>	EU272189	PoTiRb169	UKN	(Mura et al., 2008)
	<i>'Candidatus Rickettsia barbarica'</i>	NVP							
16	<i>Rickettsia bellii</i>	VP	SFG (AG)	Tick	<i>Dermacentor andersoni, Argasid and Ixodid species</i>	NR_074484.2	RML 369-C	2	(Philip et al., 1983)
17	<i>'Candidatus Rickettsia bemisiae'</i>	NVP	Torix	Whitefly	<i>Bemisia tabaci</i>	None available	no culture isolated	UKN	(Wang et al., 2020)
18	<i>Rickettsia buchneri</i>	VP	SFG	Tick	<i>Ixodes scapularis</i>	JFKF01000109	ATCC VR-1814; DSM 29016; ISO-7	1	(Kurtti et al., 2015)
19	<i>Rickettsia canada</i>	MS	SFG (AG)	Tick	<i>Haemaphysalis leporispalustris</i>	L36104	2678; ATCC VR-610	2	(McKiel et al., 1967)
	<i>Rickettsia canadensis</i>	VP							
20	<i>'Candidatus Rickettsia colombianensi'</i>	MS	SFG	Tick	<i>Amblyomma dissimile</i>	KF691750.1	Colombianensi Adcor 2	UKN	(Miranda et al., 2012)
	<i>'Candidatus Rickettsia colombiensis'</i>	NVP							
21	<i>Rickettsia conorii</i>	VP	SFG	Tick	<i>Ornithodoros moubata</i>	AF541999	ATCC VR-613; Malish	3	(Brumpt, 1932)

							7; NIAID Malish 7		
22	<i>Rickettsia cooleyi</i>	NVP	SFG	Tick	<i>Ixodes scapularis</i>	None available	no culture isolated	UKN	(Billings et al., 1998)
23	' <i>Candidatus Rickettsia culextritaeniorhynchus</i> '	MS	SFG	Mosquito	<i>Culex tritaeniorhynchus</i>	None available	no culture isolated	UKN	(Guo et al., 2016)
	' <i>Candidatus Rickettsia culicis</i> '	NVP							
24	' <i>Candidatus Rickettsia davousti</i> '	MS	SFG	Tick	<i>Amblyomma tholloni</i>	None available	no culture isolated	UKN	(Mediannikov et al., 2007)
	' <i>Candidatus Rickettsia davoustii</i> '	NVP							
25	<i>Rickettsia felis</i>	VP	SFG (TRG)	Flea	<i>Ctenocephalides felis</i>	CP000053	California 2; CNCM I- 2363; Marseille- URRWFXCal 2	2	(Bouyer et al., 2001)
26	<i>Rickettsia furnieri</i>	VP	SFG	Tick	<i>Argas lagenoplastis</i>	KF666475	AUS118; CSUR R501; DSM 28985	UKN	(Diop et al., 2018)
27	' <i>Candidatus Rickettsia gannanensis</i> '	NVP	SFG	Tick	<i>Haemaphysalis qinghaiensis</i>	None available	no culture isolated	UKN	(Yang et al., 2016)
	' <i>Candidatus Rickettsia gannanii</i> '	MS							
28	' <i>Candidatus Rickettsia goldwasserii</i> '	NVP	SFG	Tick	<i>Haemaphysalis adleri</i> and <i>Haemaphysalis parva</i>	None available	no culture isolated	UKN	(Keysary et al., 2011)
	' <i>Candidatus Rickettsia goldwasserii</i> '	MS							
29	<i>Rickettsia gravesii</i>	VP	SFG	Tick	<i>Amblyomma triguttatum</i>	DQ269434	ARRL RGBWI- 1; ATCC VR- 1664; BWI; BWI-1T; CSUR R172	2	(Abdad et al., 2017)

30	<i>'Candidatus Rickettsia haemaphysalis'</i>	NVP	SFG	Tick	<i>Haemaphysalis longicornis</i>	MG906672	ROK-HL727	UKN	(Jiang et al., 2018)
	<i>'Candidatus Rickettsia longicornii'</i>	MS							
31	<i>'Candidatus Rickettsia hebeiensis'</i>	NVP	SFG	Tick	<i>Haemaphysalis longicornis</i>	None available	no culture isolated	UKN	(Zou et al., 2011)
	<i>'Candidatus Rickettsia hebei'</i>	MS							
32	<i>Rickettsia heilongjiangensis</i>	VP	SFG	Tick	<i>Dermacentor silvarum</i>	AF178037	054; ATCC VR-1524; CSUR 054; HLJ-054	3	(Fournier et al., 2003)
	<i>'Rickettsia heilongjiangii'</i>	NVP							(Zhang et al., 2000)
33	<i>Rickettsia helvetica</i>	VP	SFG	Tick	<i>Ixodes ricinus</i>	None available	ATCC VR-1375; C3	2	(Beati et al., 1993)
34	<i>Rickettsia honei</i>	VP	SFG	Tick	first isolated from a human but suspected <i>Aponoma hydrosauri</i>	AF060705	ATCC VR-1472; RB	2	(Stenos et al., 1998)
35	<i>'Candidatus Rickettsia hongyuanensis'</i>	NVP	SFG	Tick	<i>Haemaphysalis qinghaiensis</i>	None available	no culture isolated	UKN	(Lu et al., 2022b)
36	<i>'Candidatus Rickettsia hoogstraalii'</i>	NVP	SFG	Tick	<i>Carios capensis</i>	FJ767735	Croatica; DS M 22243; UTMB 00003	UKN	(Mattila et al., 2007)
	<i>Rickettsia hoogstraalii</i>	VP			<i>Haemaphysalis sulcata</i>				(Duh et al., 2010)
37	<i>Rickettsia hulinii</i>	NVP	SFG	Tick	<i>Haemaphysalis concinna</i>	AF172942	HL-93	UKN	(Zhang et al., 2000)
38	<i>'Candidatus Rickettsia hungarica'</i>	NVP	Unknown	Tick	<i>Haemaphysalis inermis</i>	None available	no culture isolated	UKN	(Hornok et al., 2010)
39	<i>'Candidatus Rickettsia ixodis'</i>	NVP	SFG (AG)	Tick	<i>Ixodes kingi</i>	HF548205	CA10; no culture isolated	UKN	(Anstead and Chilton, 2013)
	<i>'Candidatus Rickettsia kingi'</i>	MS							
40	<i>Rickettsia japonica</i>	VP	SFG	Tick	first isolated in humans but found in <i>Haemaphysalis longicornis</i>	AP011533 (NR_074459.2)	ATCC VR-1363; YH	3	(Uchida et al., 1992)

41	' <i>Candidatus Rickettsia jiaonanensis</i> '	NVP	SFG	Tick	<i>Haemaphysalis longicornis</i>	None available	no culture isolated	UKN	(Qin et al., 2019)
	' <i>Candidatus Rickettsia jiaonani</i> '	MS							
42	' <i>Candidatus Rickettsia jingxinensis</i> '	NVP	SFG	Tick	<i>Haemaphysalis longicornis</i>	None available	no culture isolated	UKN	(Liu et al., 2016)
43	' <i>Candidatus Rickettsia johorensis</i> '	NVP	SFG	Tick	<i>Amblyomma varanense</i> and <i>Amblyomma helvolum</i>	None available	no culture isolated	UKN	(Kho et al., 2015)
44	' <i>Candidatus Rickettsia kellyi</i> '	NVP	SFG	Tick	first isolated in human, unknown vector species	None available	no culture isolated	UKN	(Rolain et al., 2006)
45	' <i>Candidatus Rickettsia khammouanensis</i> '	NVP	SFG	Tick	<i>Haemaphysalis</i> sp.	None available	no culture isolated	UKN	(Taylor et al., 2016)
46	' <i>Candidatus Rickettsia kotlanii</i> '	NVP	SFG	Tick	<i>Ixodes ricinus</i>	DQ243936	HcH1	UKN	(Sreter-Lancz et al., 2006)
47	' <i>Candidatus Rickettsia kulagini</i> '	MS	SFG	Tick	<i>Rhipicephalus sanguineus</i>	None available	no culture isolated	UKN	(Merhej and Raoult, 2011)
	' <i>Candidatus Rickettsia kulagini</i> '	NVP							
48	' <i>Candidatus Rickettsia lanei</i> '	NVP	SFG	Tick	<i>Haemaphysalis leporispalustris</i>		CA6269	UKN	(Eremeeva et al., 2018)
49	' <i>Candidatus Rickettsia laoensis</i> '	NVP	SFG	Tick	<i>Haemaphysalis</i> sp.	None available	no culture isolated	UKN	(Taylor et al., 2016)
50	' <i>Candidatus Rickettsia leptotrombidii</i> '	NVP	SFG (TRG)	Mite	<i>Leptotrombidium scutellare</i>	KX352248	no culture isolated	1	(Huang et al., 2017)
	' <i>Candidatus Rickettsia leptotrombidium</i> '	MS							
51	' <i>Candidatus Rickettsia liberiensis</i> '	NVP	SFG	Tick	<i>Ixodes muniensis</i>	None available	no culture isolated	UKN	(Mediannikov et al., 2012)
52	<i>Rickettsia lusitaniae</i>	NVP	SFG (TRG)	Tick	<i>Ornithodoros erraticus</i>	None available	PoTiRo	UKN	(Milhano et al., 2014)
53	' <i>Candidatus Rickettsia mahosoti</i> '	NVP	SFG	Tick	<i>Haemaphysalis</i> spp.	None available	no culture isolated	UKN	(Taylor et al., 2016)
	' <i>Candidatus Rickettsia mahosotii</i> '	MS							

54	<i>Rickettsia massiliae</i>	VP	SFG	Tick	<i>Rhipicephalus turanicus</i>	L36214	ATCC VR-1376; Mtu1	2	(Beati and Raoult, 1993)
55	' <i>Candidatus Rickettsia mauretanica</i> '	NVP	SFG (TRG)	Tick	<i>Ornithodoros occidentalis</i> , <i>Ornithodoros erraticus</i> , and <i>Ornithodoros normandi</i>	MN905453- MN905456	no culture isolated	UKN	(Buyse and Duron, 2020)
56	' <i>Candidatus Rickettsia mendelii</i> '	NVP	SFG (AG)	Tick	<i>Ixodes ricinus</i>	KJ882305- KJ882318	no culture isolated	UKN	(Hajduskova et al., 2016)
57	<i>Rickettsia monacensis</i>	VP	SFG	Tick	<i>Ixodes ricinus</i>	DQ100164	ATCC VR-1928; IrR/Munich	UKN	(Simser et al., 2002)
58	<i>Rickettsia mongolotimonae</i>	NVP	SFG	Tick	<i>Haemophysalis asiaticum</i>	DQ097085	HA-91	2	(Roux et al., 1997)
59	<i>Rickettsia montana</i>	MS	SFG	Tick	<i>Microtus</i> sp.? <i>Dermacentor variabilis</i>	L36215	ATCC VR-611; Tick	2	(Weiss and Moulder, 1984)
	<i>Rickettsia montanensis</i>	VP							
60	' <i>Rickettsia monteiroi</i> '	NVP	SFG	Tick	<i>Amblyomma incisum</i>	FJ269037	Intervales	UKN	(Pacheco et al., 2011)
61	' <i>Candidatus Rickettsia moyalensis</i> '	NVP	SFG	Tick	<i>Rhipicephalus appendiculatus</i>	None available	no culture isolated	UKN	(Kimita et al., 2016)
62	' <i>Candidatus Rickettsia muridii</i> '	NVP	SFG	Rodents	<i>Rhabdomys species</i> and <i>Lemniscomys rosalia</i>	None available	no culture isolated	UKN	(Essbauer et al., 2018)
63	' <i>Candidatus Rickettsia nicoyana</i> '	NVP	SFG	Tick	<i>Ornithodoros knoxjonesi</i>	KX228147	RS32	UKN	(Moreira-Soto et al., 2017)
64	' <i>Candidatus Rickettsia paranaensis</i> '	NVP	SFG	Tick	<i>Amblyomma parkeri</i>	None available	no culture isolated	UKN	(Peckle et al., 2019)
65	<i>Rickettsia parkeri</i>	VP	SFG	Tick	<i>Amblyomma maculatum</i>	U12461	Maculatum 20; NIAID maculatum 20	2	(Lackman et al., 1965)
66	<i>Rickettsia peacockii</i>	VP	SFG	Tick	<i>Dermacentor andersoni</i>	U55820	Skalkaho	1	(Niebylski et al., 1997)
67	<i>Rickettsia philipii</i>	NVP	SFG	Tick	First identified in a human then identified in <i>Dermacentor occidentalis</i>	None available	no culture isolated	2	(Grewal et al., 2014)

68	<i>'Candidatus Rickettsia principis'</i>	NVP	SFG	Tick	<i>Haemaphysalis danieli?</i> <i>Haemaphysalis japonica douglasi</i>	MG517253.1	Hjd54	UKN	(Mediannikov et al., 2006)
69	<i>Rickettsia prowazekii</i>	VP	TG	Human Lice	First identified in a human, <i>Pediculus humanus humanus</i> and <i>Pediculus humanus capitis</i>	M21789	ATCC VR-142; Brein1; Brein1	3	(Da Rocha-Lima, 1916)
70	<i>Rickettsia raoultii</i>	VP	SFG	Tick	<i>Dermacentor</i> sp	DQ365810	ATCC VR-1596; CSUR R3; Khabarovsk	2	(Mediannikov et al., 2008)
71	<i>'Candidatus Rickettsia rhabdomydis'</i>	NVP	SFG	Rodents	<i>Rhabdomys</i> spp., <i>Otomys irroratus</i> , <i>Lemniscomys rosalia</i> and <i>Aethomys chrysophilus</i>	KX944385, KX944376, KX944375	no culture isolated	UKN	(Essbauer et al., 2018)
72	<i>Rickettsia rhipicephali</i>	VP	SFG	Tick	<i>Rhipicephalus sanguineus</i>	L36216	3-7-6; Burgdorfer 3-7-female 6	2	(Weiss and Moulder, 1984, Hayes and Burgdorfer, 1979)
73	<i>Rickettsia rickettsi</i>	MS	SFG	Tick	<i>Dermacentor</i> , <i>Amblyomma</i> , <i>Rhipicephalus</i> , <i>Haemaphysalis</i>	CP018913	ATCC VR-149; Iowa; Smith	3	(Brumpt, 1922)
	<i>Rickettsia rickettsii</i>	VP				CP000766			
74	<i>Candidatus Rickettsia rioja</i>	MS	SFG	Tick	First identified in a human bitten by <i>Dermacentor marginatus</i>	None available	no culture isolated	2	(Portillo et al., 2009)
	<i>Candidatus Rickettsia riojensis</i>	NVP							
75	<i>Candidatus Rickettsia senegalensis</i>	NVP	SFG (TRG)	Flea	<i>Ctenocephalides felis</i>	KF666476	CSUR R184; DSM 2850; PU01-02; R184	UKN	(Mediannikov et al., 2015)
76	<i>Rickettsia sennetsu</i>	VP	None	raw fish (infected trematodes)	First isolated from humans, consumption of raw fish (<i>Anabas testudineus</i>) contaminated with infected trematodes	M73219	ATCC VR-367; Miyayama	2	(Misao and Kobayashi, 1956)
77	<i>'Candidatus Rickettsia sepangensis'</i>	NVP	SFG	Ticks	<i>Amblyomma varanense</i> from <i>Python molurus</i> snakes	None available	no culture isolated	UKN	(Kho et al., 2015)

78	<i>'Candidatus Rickettsia shennongii'</i>	NVP	SFG	Ticks	<i>Rhipicephalus haemaphysaloides</i>	OL855727- OL855731, OL855739	no culture isolated	UKN	(Lu et al., 2022a)
79	<i>Rickettsia sibirica</i>	VP	SFG	Tick	<i>Hyalomma</i> sp	D38628	2-4-6; ATCC VR-151; ATCC VR-541	3	(Zdrodovskii, 1948)
80	<i>'Candidatus Rickettsia siciliensis'</i>	NVP	SFG	Tick	<i>Rhipicephalus turanicus</i>	None available	no culture isolated	UKN	(Eremeeva and Stromdahl, 2011)
81	<i>Rickettsia slovaca</i>	VP	SFG	Tick	<i>Dermacentor marginatus</i>	L36224	13-B; B	2	(Sekeyova et al., 1998)
82	<i>Rickettsia tamurae</i>	VP	SFG	Tick	<i>Amblyomma testudinarium</i>	AY049981	AT-1; ATCC VR-1594; CSUR R1	1	(Fournier et al., 2006)
83	<i>'Candidatus Rickettsia tarasevichiae'</i>	NVP	SFG	Tick	<i>Ixodes persulcatus</i>	AF503168	symbiont of <i>Ixodes persulcatus</i>	UKN	(Shpynov et al., 2003)
84	<i>'Candidatus Rickettsia tasmanensis'</i>	MS	SFG	Tick	<i>Ixodes tasmani</i>	None available	no culture isolated	UKN	(Izzard et al., 2009)
	<i>'Candidatus Rickettsia tasmaniensis'</i>	NVP							
85	<i>Rickettsia thailandii</i>	NVP	SFG	Tick	<i>Ixodes granulatus</i>	None available	no culture isolated	UKN	(Kollars et al., 2001)
86	<i>'Candidatus Rickettsia thierseeensis'</i>	NVP	SFG	Tick	<i>Ixodes ricinus</i>	MT424982	no culture isolated	2	(Schötta et al., 2020)
	<i>'Candidatus Rickettsia thierseensis'</i>	MS							
87	<i>'Candidatus Rickettsia tibetani'</i>	MS	SFG	Tick	<i>Dermacentor everestianus</i> and <i>Dermacentor niveus</i>	None available	no culture isolated	UKN	(Wang et al., 2012)
	<i>'Candidatus Rickettsia tibetensis'</i>	NVP							
88	<i>Rickettsia tillamookensis</i>	VP	SFG	Tick	<i>Ixodes pacificus</i>	CP060138	CRIRC RTI001; CSUR R5043; Tillamook 23	UKN	(Gauthier et al., 2021)

89	<i>Rickettsia (Orientia) tsutsugamushi</i>	VP	ST	Mite	<i>Leptotrombidium</i> sp.	D38623	ATCC VR-150; Karp	3	(Ogata, 1931, Tamura et al., 1995)
90	<i>Rickettsia typhi</i>	VP	TG	Flea	<i>Ctenocephalides</i> sp.?	U12463	ATCC VR-144; Wilmington	3	(Philip, 1943)
91	' <i>Candidatus Rickettsia uilenbergii</i> '	MS	SFG	Tick	<i>Amblyomma tholloni</i>	None available	no culture isolated	UKN	(Mediannikov et al., 2007)
	' <i>Candidatus Rickettsia uilenbergii</i> '	NVP							
92	' <i>Candidatus Rickettsia uralica</i> '	NVP	SFG	Tick	<i>Ixodes persulcatus</i> , <i>Ixodes trianguliceps</i>	KP747669	no culture isolated	UKN	(Ilgolkina et al., 2015)
93	' <i>Candidatus Rickettsia vini</i> '	NVP	SFG	Tick	<i>Ixodes ricinus</i> and <i>Sylvia atricapilla</i>	None available	4GA09/32	UKN	(Palomar et al., 2012)
94	' <i>Candidatus Rickettsia wissemanii</i> '	NVP	SFG	Tick	<i>Ornithodoros hasei</i>	LT558851	G1329	UKN	(Tahir et al., 2016)
95	' <i>Candidatus Rickettsia xinyangensis</i> '	NVP	SFG	Tick	Originally identified in humans <i>Haemaphysalis longicornis</i>	KY617772	no culture isolated	2	(Li et al., 2020)
96	<i>Candidatus Rickettsia yingkouensis</i>	NVP	SFG (TRG)	Mosquitoes	<i>Anopheles pullus</i> , <i>Anopheles sinensis</i>	None available	no culture isolated	UKN	(Lu et al., 2023)

Abbreviations : VP – validly published, NVP – not validly published; MS – misspelling. SFG – spotted fever group; TG – typhus group; TRG – transitional group; AG – ancestral group; ST – scrub typhus; UKN - Unknown.

1.2 AIMS AND OBJECTIVES

The aims of this study are:

- To evaluate commercial assays and the diagnostic testing available for *Rickettsia* in South Africa,
- To characterize the *Rickettsia* associated with severe cases of tick bite fever (TBF) in South Africa, and
- To investigate the presence of *Rickettsia* species in ectoparasitic vectors collected from different sites in South Africa.

The specific objectives include:

Part 1

- To evaluate commercially available serology tests for *Rickettsia* in South Africa using a panel of clinical samples, and
- To conduct an inter-laboratory comparison (ILC) to investigate the performance of laboratories in South Africa offering routine serological tests for *Rickettsia*, and identify capacity and gaps.

Part 2

- To perform molecular sequencing to type the Rickettsial species involved in severe cases of TBF in South Africa, and
- To screen ticks collected from different sites in South Africa for the presence and genetic diversity of *Rickettsia*.

1.3 SCOPE OF THE STUDY

Rickettsias are a fascinating group of organisms said to fit in somewhere between viruses and bacteria. They are found globally and cause a plethora of diseases in humans, animals and involve invertebrate vectors e.g. lice infected with *R. prowazekii*, as well as arthropod hosts. Rickettsial pathogenicity is variable with a broad spectrum of associated manifestations of variable severity ranging from mild symptoms, e.g. *R. slovaca*, to diseases with a high rate of case fatality e.g. *R. rickettsia* (Blanton, 2019). It has even been shown that some rickettsias (particularly SFG rickettsias), may cause seroconversion with little or no recognized illness (Blanton, 2019). Rickettsias are becoming increasingly recognised globally. With the recognition of these organisms, is the need for clinicians to have increased awareness and recognition of the spectrum of diseases caused by these organisms (Blanton, 2019, Azad and Beard, 1998).

The worldwide public health impact of rickettsial infections is assumed to be quite high although the impact on the infected or loss of productivity is largely unmeasured (Mahajan, 2012). It has been suggested that every rickettsial species has potential for human pathogenesis if its arthropod reservoir is capable of biting humans (La Scola and Raoult, 1997). The scope and importance of recognized tick-associated rickettsial pathogens has increased considerably in the past two and a half decades. A number of tick-borne rickettsiae species previously considered non-pathogenic, are now been linked with human infections, whilst new *Rickettsia* species of undetermined pathogenicity are frequently being detected in or isolated from ticks around the world (Parola et al., 2013).

In South Africa, rickettsiology has come a long way from some now seemingly questionable practices of the 1940s and 1950s involving the testing of vaccines on disadvantaged persons, the application to people of dichlorodiphenyltrichloroethane (DDT) to cleanse them of lice, and diagnostics that relied heavily on clinical presentation and the Weil-Felix (WF) test (Gear, 1954). Rickettsioses are medically important emerging infectious diseases in southern Africa, where a marked increase in the number of cases has been observed among both residents and tourists (Raoult et al., 2001,

Pretorius and Birtles, 2004). In recent years, at least four (4) *Rickettsia* species that have been associated with human disease in South Africa, namely *Rickettsia conorii* (recognized as the agent of Mediterranean spotted fever (MSF), *R. africae* (identified as the agent of African tick-bite fever (ATBF), *R. aeschlimannii* (first case reported in 2002) and *R. mongolotimonae* (reported in a 34-year-old, HIV-seronegative, construction worker, working near Ellisras) (Pretorius and Birtles, 2004). African tick bite fever is currently the most common rickettsiosis in sub-Saharan Africa and should be considered as a differential diagnosis for patients with fever and/or exanthema returning from or living in southern Africa (Portillo et al., 2007). Rickettsial diseases generally present with non-specific symptomologies such as fever, myalgia and headache, that are consistent with a range of other febrile illnesses, and thus clinical diagnosis may be quite difficult. Although some AFTB cases may present with a distinct vesicular cutaneous rash, for *R. africae* this is not a common characteristic of infection (Raoult et al., 2001, Kelly et al., 1992, Kemp et al., 2014). It is suspected that tick-bite fever is underreported, as many people experiencing flu-like illness will not seek medical evaluation and will thus be missed (Kemp et al., 2014). Initial TBF diagnosis is often based solely on symptoms and case history since confirmation in the first week of illness is challenging due to low sensitivity of both PCR and serology (Kemp et al., 2014).

In South Africa, the most commonly used diagnostic technique for rickettsial infections is serology. *Rickettsia* testing is offered at various public and private laboratories throughout the country, and the Centre for Emerging Zoonotic and Parasitic Diseases (CEZPD) at the National Institute for Communicable Diseases (NICD) acts as the national reference laboratory for diagnosis of arboviral, bacterial and viral haemorrhagic fevers, with rickettsiae being among many pathogens dealt with by the centre (Kemp et al., 2014). The CEZPD-NICD performs IFAs instead of the outdated WF test due to increased sensitivity and specificity. The NICD also conducts molecular detection and identification by polymerase chain reaction (PCR). The gold standard for the diagnosis of rickettsias remains isolation and cell culture and the CEZPD-NICD is fully equipped to safely carry out these procedures (Kemp et al., 2014).

Rickettsial disease, commonly presenting in South Africa as TBF, is a very common cause of febrile illness in the country. Seroprevalence levels in selected communities approach 80% (data source: NICD, unpublished). A 2014 seroprevalence study conducted in the Bushbuckridge District of Mpumalanga Province, South Africa (Quan et al., 2014) reported a rate of 20% (13/65) recent 57% (33/58) past TBF infections present in patients presenting with acute febrile illness at a nearby clinic, and a seroprevalence of 92% (59/64) in farmworkers and veterinary staff in the area. Furthermore, TBF is also a commonly-reported disease in travellers to South Africa. Nevertheless, TBF is a neglected disease with diagnosis often based on clinical assessments, opening the door for mis- and under-diagnosis of what is sometimes a life-threatening disease. Contributing to the latter is the lack of rapid and accurate laboratory tests for the diagnosis of TBF.

CHAPTER 2 – LITERATURE REVIEW

2.1 INTRODUCTION

Rickettsia, first described by da Rocha Lima in 1916, describes an ever-expanding group of obligate, intracellular organisms (Abdad et al., 2018, Ricketts, 1909). In early reports, however, the term ‘rickettsiae’ was used to describe a diverse group of bacteria with the only commonality being their intracellular lifestyle (Diop et al., 2020). ‘*Rickettsia*’ belongs to the family *Rickettsiaceae*, within the order Rickettsiales (also said to contain the families *Bartonellaceae* and *Anaplasmataceae*) (Diop et al., 2020). The transmission of rickettsiae is primarily associated with vectors including ticks, fleas, mites and lice that feed on human or animal hosts (Abdad et al., 2018, Fournier and Raoult, 2009, Anstead and Chilton, 2013, Blanton, 2019). Although not considered primary transmitters, *Anopheles* mosquitoes (*Anopheles gambiae*, *Anopheles albopictus*, *Anopheles sinensis* and *Anopheles pullus*) (Guo et al., 2016, Dieme et al., 2015, Berrang-Ford et al., 2012, Lu et al., 2023) and *Culex* mosquitoes (*Culex tritaeniorhynchus*) (Guo et al., 2016) have also been reported as potential *Rickettsia* species transmission vectors. It is anticipated that many more hosts, vectors and *Rickettsia* species will be discovered as researchers seek them and humans continue to travel into previously uninhabited areas. Through the development of molecular techniques and over a century of investigation into this group of organisms, the phylogeny and taxonomy of rickettsias still remains a topic of debate amongst expert rickettsiologists (Fournier and Raoult, 2009). The genus *Rickettsia* currently comprises 143 child taxa; 29 of which are validly published with correct names and 34 of which have validly published names, including synonyms (<https://www.bacterio.net> accessed: 12/07/2023) (Table 2.1), and this number is expected to continue to increase due to the exponential technological advances of molecular techniques and the enormous decrease in costs associated with use of these techniques and tools (Abdad et al., 2018, Fournier and Raoult, 2009).

Rickettsioses present in a number of diseases or syndromes depending on the rickettsial species responsible for the infection (Binder et al., 2019). Practiced physicians and

epidemiologists often experience challenges in consistent and accurate confirmation or exclusion of rickettsioses resulting in both under-diagnosis and over-diagnosis of certain rickettsial diseases (McClain and Sexton, 2019). Disease can present as mild flu-like illness that is self-limiting and requires no treatment, to severe and life-threatening disease or even fatal outcomes, particularly when highly pathogenic species such as *Rickettsia rickettsii* and *Rickettsia prowazekii* are implicated in the infection and there is a delay in diagnosis and treatment (Blanton, 2019).

Table 2.1 Taxonomic nomenclature of *Rickettsia* genus child taxa as presented on the List of Prokaryotic Names with Standing Nomenclature website (<https://psn.dsmz.de/> accessed: 11/11/2021)

Row Labels	Count of No
Not validly published	71
Not validly published, basonym of name in Approved Lists	9
Orthographic variant (inaccurate spelling)	29
Validly published under the ICNP ⁱ	33
Validly published under the ICNP, conserved name	1
Grand Total	143

i. ICNP - International Code of Nomenclature of Prokaryotes

In this chapter, the classification of *Rickettsia* species as it currently stands will be outlined; thereafter, the epidemiology and clinical disease manifestations will be described and in conclusion of this review chapter the clinical diagnostics and available treatments will be discussed.

2.2 STRUCTURE, CLASSIFICATION AND ANTIGENIC TYPES

2.2.1 GENOME AND CLASSIFICATION OF RICKETTSIA SPECIES

The order Rickettsiales was historically divided into three (3) families, namely; *Rickettsiaceae*, *Bartonellaceae*, and *Anaplasmataceae* (La Scola and Raoult, 1997). This

has recently changed and the order Rickettsiales, α -Proteobacteria now only comprises of two (2) families, *Rickettsiaceae*, which include the genera *Orientia* and *Rickettsia*; and *Anaplasmataceae*, predominantly comprising of arthropod commensals of the genera *Ehrlichia*, *Wolbachia*, *Anaplasma* and *Neorickettsia* (Diop et al., 2020, Emelyanov, 2001, Fournier and Raoult, 2009, Darby et al., 2007, Valbuena and Walker, 2009). Within the family *Rickettsiaceae*, the genus *Orientia* is represented by numerous strains of the single species *Orientia tsutsugamushi* (Emelyanov, 2001), but this is not so for the genus *Rickettsia*, which currently comprises dozens of species that have been isolated from, or detected in ticks alone. Many of these species are incompletely described and/or uncultivated species. Rickettsiales taxonomy is very complex and continues to be updated (Mahajan, 2012, Abdad et al., 2018, Fournier and Raoult, 2009, Merhej et al., 2011). The advent of 16S ribosomal ribonucleic acid (rRNA) gene analysis has allowed researchers to better study the phylogenetic relationships among members of the order Rickettsiales and has provided some resolution about the inter-relatedness of the families and species within it. Through molecular techniques, it has been found that *Coxiella burnetii* (previously classified within the family) is quite distinct from other rickettsiae (i.e. α -subgroup of Proteobacteria) and bears closer similarity to γ -subgroup (Abdad et al., 2018), consequently being removed from the family. Similarly, the genus *Rochalimaea* was amalgamated with the *Bartonella* genus and thus excluded from the order Rickettsiales, due to its members phylogenetically classifying as α -2 subgroup Proteobacteria (Shpynov et al., 2018).

The absence of an official, universally accepted set of rules for determining whether a species should be included into the *Rickettsia* genus has been a hindrance in *Rickettsia* phylogeny and taxonomy (Fournier and Raoult, 2009, Merhej et al., 2011). Phylogenetic studies of rickettsiae were, until the 1990s, based on highly unreliable criteria, namely the assessment of morphological, antigenic, and metabolic characteristics, as well as by biological features like intracellular localization, ideal proliferation temperature, and cross-reactivity of the patient's infected serum with the somatic antigens of three (3) *Proteus* strains (Abdad et al., 2018, Fournier and Raoult, 2009). The long-standing reference method for rickettsial species classification is mouse serotyping, a taxonomic tool

developed by Philip in 1978 (Diop et al., 2020). Mouse serotyping required acute phase mouse sera of an experimentally infected mouse to be subjected to an IFA that would detect *Rickettsia* species-specific epitopes of the surface-exposed S-layer proteins (rOmpA and rOmpB), and the Sca4 (PS120) protein (Fournier and Raoult, 2009). Although this method has its merits, it is very laborious as it requires each novel isolate to be compared with all formerly defined species and lacks both intra- and inter-laboratory reproducibility (Diop et al., 2020, Fournier and Raoult, 2009).

The creation of a novel *Rickettsia* species currently relies on a multiphasic inquisition providing phylogenetic classification, multilocus sequence typing (MLST) results and the description of phenotypic data, particularly human pathogenesis, geographic distribution and arthropod associations. Validation of a species name is only possible following the submission of type strains into at least two (2) official culture collections in different countries and through publication in a validation list in the *International Journal of Systematic and Evolutionary Microbiology* (Diop et al., 2020). Additionally, the currently-accepted guidelines, i.e. the use of 16S rRNA gene sequence similarity, genomic guanine and cytosine (G+C) content and deoxyribonucleic acid (DNA)–DNA hybridization (DDH) relatedness, which are widely accepted for the description of extracellular bacteria, cannot accommodate strictly intracellular bacteria and are therefore not applicable to *Rickettsia* species (Fournier and Raoult, 2009, Diop et al., 2020, Fournier et al., 2003). Essentially, the accepted molecular criteria used to classify bacterial isolates include a DDH of greater than (>) 70%, a G+C content differences of less than (<) 5% and a 16S rRNA divergence of < 3%. These criteria do not apply to *Rickettsia* species since DDH values of >73% would classify *Rickettsia conorii*, *Rickettsia sibirica*, *Rickettsia rickettsii*, and *Rickettsia montanensis* as a single species; DNA G+C content values of 32–33% would classify all SFG as one species and DNA G+C content value of 29% would classify all TG rickettsiae as second species; and all rickettsiae would classify as a single species with a 16S rRNA divergence of <2% (Fournier and Raoult, 2009, Diop et al., 2020). The average nucleotide identity (ANI) method, an alternate technique to DDH used to describe a novel bacterial species, is also considered unsuitable for identifying *Rickettsia* species since these criterion, ANI values of > 94% would identify *R. conorii*, *R. prowazekii*,

R. rickettsii, *R. sibirica* and *Rickettsia typhi* as one species (Konstantinidis and Tiedje, 2005, Fournier and Raoult, 2009). These problems have led to the development of new taxonomic methods for classifying rickettsiae.

A number of genome-sequencing efforts have been directed towards the genus with the aim of better understanding molecular mechanisms fundamental to the evolution and pathogenicity of rickettsias (Merhej and Raoult, 2011, Shpynov et al., 2018). Whole-genome sequencing (WGS), first introduced in 1995, has led to consistent progress in the output, cost reduction and availability of DNA sequencing technologies allowing for complete genomic sequences to be obtained and used as a source of taxonomic information for prokaryotes (Diop et al., 2020). Modern classification is increasingly being based on whole-genome analysis data (Guillemi et al., 2015, Fuxelius et al., 2007, Abdad et al., 2018, Fournier and Raoult, 2009). Sequence data are currently available for several genes for the majority of recognized rickettsial isolates, inclusive of the panbacterial 16S rRNA (*rrs*) (Barrs et al., 2010) and citrate synthase (*gltA*) genes and the *Rickettsia*-specific *ompA* and *ompB* genes and *gene D*, encoding the surface-exposed, high-molecular-weight proteins rOmpA, rOmpB, and PS120, respectively (Fournier et al., 2003). The practicality of sequencing several genes for taxonomic determinations has been demonstrated and the *Ad Hoc Committee for the Re-Evaluation of the Species Definition in Bacteriology* has highlighted the necessity to sequence at least five (5) genes, inclusive of protein-coding genes (Fournier et al., 2003).

Rickettsial genomes, when compared to those of free-living bacteria, exhibit some atypical properties. It has been revealed through genomic content comparisons that gene loss is a key and continuous course for the evolution of all lineages (Merhej and Raoult, 2011, Diop et al., 2020). There is significant intra-genus size variability (1.1–1.5 Mb) exhibited by gene content (about 900–1500 genes), which is perhaps a reflection of the significant range of rickettsial hosts and infection approaches (Merhej and Raoult, 2011). *Rickettsia* species have small genomes ranging from 1.11 Mb to 2.1 Mb, with a low G-C content (29 % to 33 %) for *R. typhi* and “*Rickettsia* endosymbiont of *Ixodes scapularis*”

respectively (Merhej et al., 2014, Parola et al., 2013, Blanton, 2019). It is hypothesized that the small genome size has developed as a result of the decline of formerly larger genomes, adapting to parasitic/symbiotic lifestyles (Blanc et al., 2007, Diop et al., 2020). Gene decay is said to be the mechanism of gene reduction, with numerous pseudogenes and a large proportion of noncoding DNA (Walker, 2007, Amiri et al., 2003). Transitioning from free-living existence to close symbiosis with eukaryotic cells is frequently observed in bacterial evolution, reportedly having occurred in *Mycoplasma*, *Phytoplasma*, *Chlamydia*, and the α - and γ -proteobacteria (Blanc et al., 2007). Rickettsias have highly conserved genomes, with comparable gene content and synteny (McLeod et al., 2004, Walker, 2007, Diop et al., 2020). The genes encoding for the enzymes responsible for sugar metabolism, lipid biosynthesis, nucleotide synthesis, and amino acid synthesis, and despite their ability to synthesize adenosine tri-phosphate (ATP), seem to also have been lost and therefore leading to rickettsiae being considered energy parasites (Yu et al., 2009). A unique process to *Rickettsia* and *Chlamydia*, another genus of intracellular bacteria, species is the ATP/ADP (adenosine di-phosphate) translocase transport system which relocates ATP from the host cell cytoplasm into the bacteria. Their nutrient-rich (amino acids and nucleotides) cytosolic niche has encouraged rickettsiae to abandon the enzyme-encoding genes for sugar metabolism and for lipid, nucleotide, and amino acid synthesis. It is probably this characteristic that is presumably accountable the inability to culture them in cell-free medium (Walker, 2007).

Rickettsia pathogenicity, molecular data and several other phenotypic characteristics have led to their classification into four groups or clades (De Vito et al., 2020): SFG, i.e. species transmitted by predominantly hard ticks such as *R. conorii*, and *R. rickettsia* (Shpynov et al., 2018); TG, which includes *R. typhi* and *R. prowazekii* (Shpynov et al., 2018); ancestral group (AG) that classifies the divergent species *Rickettsia bellii* and *Rickettsia canadensis* and considered the only group to be non-pathogenic and incapable of causing human disease (Blanton, 2019); and transitional group (TRG) comprising *Rickettsia australis* (Brouqui et al., 2004), *Rickettsia felis* (flea-borne) and *Rickettsia akari* (louse-borne) that exhibit characteristics common to both SFG and TG species (Fournier and Raoult, 2009, Valbuena and Walker, 2009, Emelyanov, 2001,

Macaluso and Paddock, 2014, Brown and Macaluso, 2016, Guillemi et al., 2015) (Table 1.1). The “ancestral” group classification for *R. bellii* and *R. canadensis* appeared to branch outside SFG and TG indicating a divergence preceding the rift between the SFG and the TG, but since neither *R. bellii* and *R. canadensis* are truly ‘ancestors’ of other *Rickettsia* species, the terminology ‘ancestral’ group was considered unjustified and could not be used going forward (Fournier and Raoult, 2009). It was additionally determined, based on genome phylogeny, that there was insufficient evidence to warrant the establishment of a separate group for *R. felis*, *R. akari* and *R. australis*, therefore ‘transitional’ group *Rickettsia* species should be considered as SFG members (Fournier and Raoult, 2009). Consequently, *Rickettsia* are classified primarily as SFG or TG (Binder and Gupta, 2015).

2.2.2 MORPHOLOGICAL AND BIOLOGICAL CHARACTERISTICS

Rickettsia, previously considered a ‘catch-all’ term for small and uncultivable bacteria otherwise unidentified, is now solely applicable to small (0.3-0.5 by 0.8-2.0 µm), obligately intracellular, Gram-negative arthropod-borne coccobacilli that retain basic fuschin when stained by the Gimenez method (Brouqui et al., 2004, Fournier and Raoult, 2009, Blanton, 2019). They grow freely within eukaryotic cells and divide by binary fission in the cytoplasm (Fournier and Raoult, 2009). Typically, rickettsiae have a periplasmic layer separating the inner bilayer and outer membranes (Fang et al., 2017) and reside within the host cell cytosol (Blanton, 2019). These eukaryotic cell-infecting bacilli, are free-living in host cell cytoplasm, multiplying and utilizing the host cell’s ATP, amino acids, and phosphorylated sugars with their active transport systems (Blanton, 2019). Host cell infection typically causes disease in vertebrate, and in some instances, the invertebrate hosts that act as reservoirs and transmitters of *Rickettsia* species (Fournier and Raoult, 2009, Emelyanov, 2001, Valbuena and Walker, 2009, Fang et al., 2017). *Rickettsia* species have several major antigenic regions targeted by antibodies produced by infected humans, specifically the lipopolysaccharide (LPS) and outer membrane proteins (Omp) of the surface cell antigen (SCA) family, typically the 120-kDa S-layer protein (OmpB or Sca5), ribosomal OmpA (rOmpA) or Sca0 (only existing in SFG rickettsiae), and Sca4 (Fournier and Raoult, 2009).

Rickettsia prowazekii infiltrates the targeted cell by induced phagocytosis, and rapidly dodges the phagosomes present in the cytosol (Figure 2.1). Phospholipase enzymes are naturally involved in both entrance and egress of parasites, but the extent of involvement remains currently unknown. Irrespective of whether the bacterial enzyme or rickettsial-induced host phospholipase activity, it is thought that the expression of this activity during close contact between parasite and host is aligned with rickettsial capability for growth within intact, living cells (Emelyanov, 2001).

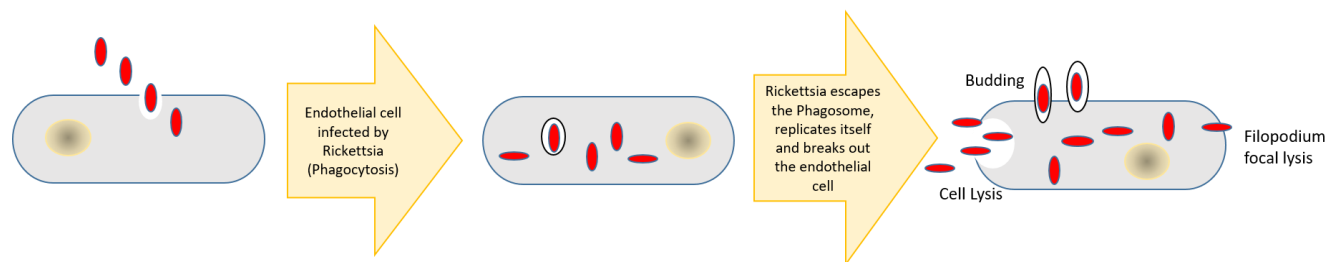


Figure 2.1. *Rickettsiae* infecting an endothelial cell, propagating, and escaping the cell through cell lysis, budding or filopodium focal lysis

2.3 EPIDEMIOLOGY

2.3.1 GEOGRAPHICAL DISTRIBUTION

The majority of rickettsial species are tied to particular areas primarily due to the environmental, weather and natural host range limitations (Abdad et al., 2018). Nevertheless, certain rickettsiae (e.g. *R. felis* and *R. typhi*) are found worldwide (Abdad et al., 2018). Ticks being the primary vector, it stands to reason that changes in tick distribution may cause an acceleration in the transmission of tick-borne rickettsioses and other emerging zoonotic diseases globally (Liu et al., 2016, Blanton, 2019). Rickettsias have been found on every continent except Antarctica (Figure 2.2) (Abdad et al., 2018) and as widespread travel continues to increase, so too will the scourge of vector-borne diseases including rickettsioses (Binder and Gupta, 2015, Blanton, 2019). Tick-borne disease in high risk areas are not necessarily static, with spatial and temporal margins of risk for a given TBD fluctuating, as preferable environments increase or decrease (Fritz,

2009, Swei et al., 2020). Transitory climatological conditions (e.g. a wet, mild winter) in endemic areas can spread tick activity and protracted weather changes can render previously non-endemic areas into ideal tick habitats (e.g. warmer temperatures at higher altitudes or upper latitudes). Likewise, spatial dimensions of risk areas may be physically altered through human encroachment or changes to existing tick habitats (Fritz, 2009).

South Africa welcomes well over eight million local and international tourists annually, owing to its unique wildlife and eco-tourist attractions (Tarragona et al., 2021). This eco-tourism inevitably increases the likelihood of contact between animals and humans, and indeed contact with the vectors responsible for zoonotic diseases (Tarragona et al., 2021, Jensenius et al., 2003b, Jensenius et al., 2003a). There are at least 14 SFG rickettsiae that have reportedly occurred in Africa, including *R. aeschlimannii*, *R. africae*, 'Candidatus *R. asemboensis*', *R. argasii*, *R. conorii*, *R. helvetica*, *R. hoogstraalii*, *R. massiliae*, *R. monacensis*, *R. raoultii*, 'Candidatus *R. siciliensis*', *R. sibirica mongolitimonae*, and *R. slovaca* (Adjou Moumouni et al., 2016, Blanton, 2019, Luce-Fedrow et al., 2015). Several of these species produce disease pathognomically similar to human infection with *R. conorii* – the agent of MSF, and *R. africae* – the agent of ATBF, the two (2) species most frequently associated with human infection on the continent (Binder and Gupta, 2015, Adjou Moumouni et al., 2016, Jensenius et al., 2003a). In the past, *R. conorii* was considered the only tick-borne rickettsial infection in Africa, causing both severe and milder forms of MSF (Raoult et al., 2001, Parola et al., 2001, Fournier et al., 2002). *Rickettsia conorii* is widely distributed around the Mediterranean Sea (Algeria, Egypt, Libya, Morocco, and Tunisia) and has also been isolated and/or detected in Central Africa, Kenya, SA, and Zimbabwe (Parola et al., 2001, Luce-Fedrow et al., 2015). *Rickettsia africae* is generally carried by *Amblyomma* species ticks and distributed throughout sub-Saharan Africa with over 20 sub-Saharan African countries reportedly detecting it in humans and ticks (Binder and Gupta, 2015, Adjou Moumouni et al., 2016). Human infection with *R. africae* was first reported in a Zimbabwean patient in 1992 (Parola et al., 2001, Toutous-Trellu et al., 2003). After its recognition, there were numerous cases of ATBF reported in tourists returning from southern Africa (Jones, 2005, Jensenius et al., 2006, Toutous-Trellu et al., 2003, Walker, 2003, Raoult et al., 2001,

Johnson et al., 2019) including a report of a *R. africae* outbreak in thirteen (13) participants of an adventure race (Fournier et al., 1998). African tick bite fever is still considered the predominant rickettsial disease in sub-Saharan Africa (Jensenius et al., 2004a).

Typhus group rickettsiae, i.e. *R. prowazekii* and *R. typhi*, are transmitted by lice and fleas (*Xenopsylla cheopis* and *Ctenocephalides felis*, respectively in some parts of the United States of America in a cycle involving opossums) and are globally distributed since fleas are found on all continents, including Antarctica. Furthermore, fleas are adapted to a wide range of environments and hosts, including equatorial deserts, tropical rainforests and arctic tundra (Bitam, 2012, Legendre and Macaluso, 2017). Murine typhus, caused by *R. typhi*, mainly occurs in warm climates worldwide, especially in coastal regions and port cities where there are rodents present. This rickettsiosis is widespread in European countries like Portugal, Spain, and Greece (Blanco and Oteo, 2006).

Rickettsia felis, harbored and transmitted by the cat flea (*C. felis*), is the causative agent of emerging flea-borne spotted fever (FBSF). Human disease attributed to *R. felis* was first reported in a Texan patient in 1994 and has since been reportedly infecting humans globally (Legendre and Macaluso, 2017). In Africa, recent studies have linked *R. felis* with around 15% of patients with febrile illness of unidentified origin demonstrating PCR-detectable levels of *R. felis* in their blood and has been reported throughout Africa: in Algeria, the Democratic Republic of the Congo, Ethiopia, Egypt, Gabon, Ivory Coast, Morocco, Kenya, Senegal and Tunisia (Legendre and Macaluso, 2017). A notable association demonstrated between *R. felis* and human disease in Africa was reported in an eight (8)-month-old Senegalese girl presenting with vesicular fever (Legendre and Macaluso, 2017).

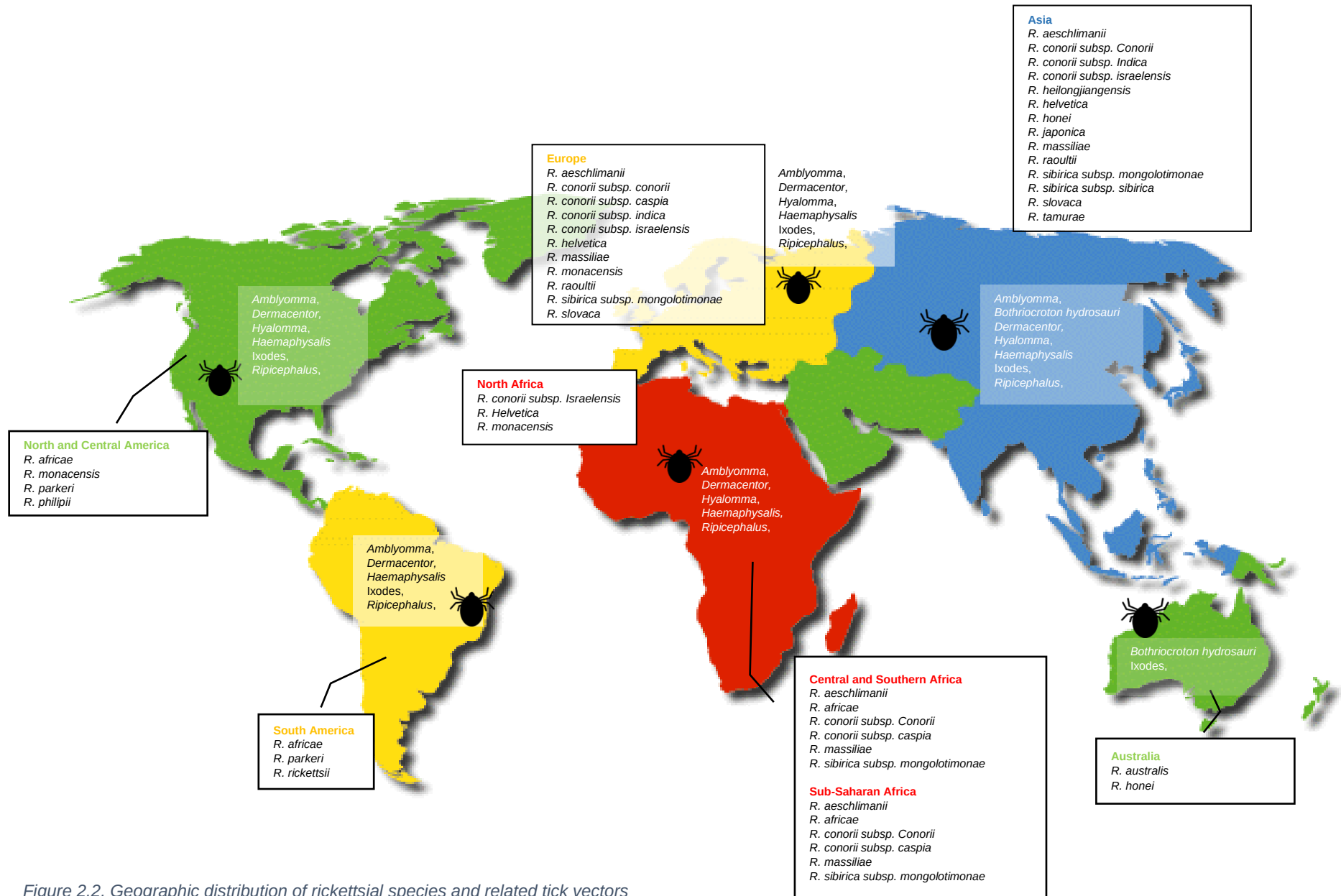


Figure 2.2. Geographic distribution of rickettsial species and related tick vectors

2.3.2 TRANSMISSION

Some of the worst plagues in history have been vector-borne diseases, examples of which include the Black Death caused by *Yersinia pestis* in 14th century Europe and yellow fever epidemics in the Americas (Chitanga et al., 2014). A consistent decline in vector-borne diseases was observed in the 20th century with the initiation of widespread vector control programs; however, a number of these diseases have reemerged in the 21st century, presumably due to international developments in animal husbandry, urbanization, contemporary transportation and globalization (Chitanga et al., 2014, Tarragona et al., 2021, Swei et al., 2020). Tick-borne diseases (TBD) are among these reemerging vector-borne diseases (Chitanga et al., 2014, Madison-Antenucci et al., 2020). The lifecycle of *Rickettsia* species is dependent on survival in small mammal hosts that serve as reservoirs or amplifiers, and in arthropods (e.g. ticks, fleas, lice, and mites) which are often responsible for vectoring the transmission of rickettsias. Excluding *R. prowazekii*, humans are usually incidental hosts (Faccini-Martinez et al., 2014). As mentioned previously, ticks, particularly hard (Ixodid) ticks, are responsible for transmission of SFG rickettsiae in their saliva during biting and feeding (Abdad et al., 2018). This mode of transmission is also applicable to rickettsia-harboring mites. Rickettsiae harbored by fleas and lice are transmitted by vector fecal material entering the host at bite sites or via skin abrasions or cuts in the skin, particularly during scratching of the itching bite-sites (Abdad et al., 2018). Typhus group rickettsiae are sometimes transmitted through the inhalation of aerosolized contaminated dust particles (Abdad et al., 2018).

In sub-Saharan Africa, *R. africae* and *R. conorii* are the two (2) main rickettsial species circulating in the tick species endemic to the region. *Rickettsia africae*, in particular, is reported to be vector specific, almost exclusively circulating in the aggressive *Amblyomma* genus; specifically, *Amblyomma variegatum* (the tropical bont tick) in the sub-Saharan area and *Amblyomma hebraeum* (the southern African bont tick) in SA (Jensenius et al., 2003a, Jensenius et al., 2003b, Cowan, 2000, Althaus et al., 2010, Fournier et al., 2009, Uslan and Sia, 2004, Walker and Ismail, 2008). There have however been reports of *R. africae* being detected in *Hyalomma dromedarii* (Kernif et al., 2012, Bitam, 2012) and *Rhipicephalus* species (Bitam, 2012, Portillo et al., 2007) although this was not conclusively proven (Chitanga et al., 2014). *Rickettsia africae* is

recognized as one of the most common causes of rickettsiosis in travelers returning to their countries from sub-Saharan Africa (Walker, 2003, Uslan and Sia, 2004, Tsai et al., 2008). Second to malaria, ATBF has the highest frequency of identification as the cause for systemic febrile illness in travelers (Prado et al., 2017, Binder and Gupta, 2015, Delord et al., 2014). *Rickettsia africae* has also been shown to occur on Caribbean islands where *A. variegatum* ticks, introduced to the region from Africa, have become established (Walker, 2003). *Rickettsia africae* is reportedly present in approximately 50 % of *Amblyomma* ticks, representing a high contamination rate with humans considered an accidental host (Althaus et al., 2010, Kelly et al., 1991). *Rickettsia* species in ticks will be examined and discussed in a later chapter of this thesis. The SFG *Rickettsia* species (like *R. conorii*, *R. parkeri*, *R. rickettsia*, *R. africae* and several others), are mainly transmitted by 'hard ticks' (Acari: Ixodidae, particularly the genera *Amblyomma*, *Dermacentor*, and *Rhipicephalus*) in any developmental phase (adult, nymph, and larva), and are amongst the oldest recognized vector-borne zoonoses (Cowan, 2000, Emelyanov, 2001, Fournier and Raoult, 2009, Valbuena and Walker, 2009, Blanton, 2019).

Insect-borne rickettsiae have, throughout history, been significantly prominent zoonotic pathogens in human populations globally, from louse-borne epidemic typhus reportedly affecting Napoleon's armies retreating from Moscow, to the modern flea-borne endemic typhus reemergence in southern United States (Brown and Macaluso, 2016). Interestingly it is said that louse-borne typhus epidemics have resulted in higher fatality rates than all the wars in combination (Azad and Beard, 1998). Unlike the ticks that transmit SFG rickettsiae, TG rickettsiae are mainly transmitted by insect vectors (Valbuena and Walker, 2009). The association of TG rickettsiae with hematophagous insects like human body lice (*Pediculus humanus*) and rodent fleas (e.g. *X. cheopis*) aids the rapid spread of rickettsiae in susceptible populations (Azad and Beard, 1998). Intermittent feeders, such as fleas and human body lice, are adept to multiple feedings and consequent *Rickettsia* species transmission to numerous hosts (Azad and Beard, 1998). Rapid *R. prowazekii* human-to-human transmission by infected lice can result in epidemic typhus outbreaks (Azad and Beard, 1998, Merhej and Raoult, 2011).

Rickettsia prowazekii, the agent of epidemic typhus, is spread by human body lice (*Pediculus humanus* and *Pediculus humanus humanus*), and unlike ticks that are capable of transovarial transmission (TOT), *R. prowazekii*-infected lice usually die within one (1) to three (3) weeks of consuming infected blood meal (Valbuena and Walker, 2009, Houhamdi et al., 2002, Merhej and Raoult, 2011). Body lice live inside clothing and contract the infection whilst feeding on rickettsaemic patient blood (Blanton, 2019). Lice are not adapted to raised body temperatures so when an infected patient becomes febrile, the lice migrate to a new host and consequently spread the infection (Blanton, 2019). Louse-borne rickettsiae are generally considered pathogenic to lice, since the bacilli rapidly multiply in the anterior gut epithelial cells causing the infected epithelial cells to expand and ultimately burst due to over-replication. This results in the rickettsiae being released into the gut lumen and ultimately the feces (Houhamdi et al., 2002, Merhej and Raoult, 2011, Mahajan, 2012). Only louse gut epithelial cell lining is infected; therefore *R. prowazekii* is not spread through direct introduction into the host's bloodstream since louse salivary glands are not infected (Valbuena and Walker, 2009). The infected lice frequently appear red as ingested blood is released into the haemocoel. Rickettsial transmission to vertebrates take place when infected insect feces is inoculated at the bite site during feeding. Lice can transmit the rickettsiae to a single host, or to other lice through horizontal transmission (Houhamdi et al., 2002, Merhej and Raoult, 2011, Mahajan, 2012). There is limited proof available supporting the concept of vertical transmission of *R. prowazekii* in human body lice, since the infected lice perish due to obstruction of alimentary tract (Mahajan, 2012). Generally, person-to-person transmission of louse-borne rickettsiae requires close, personal contact where lice is transferred, but it has been suggested that air-borne transmission may occur through inhalation of dried louse feces. This is particularly a concern for people living in crowded conditions with poor hygiene and sanitation (Mahajan, 2012).

Most *Rickettsia* species are seemingly non-detrimental to arthropod hosts, despite being able to multiply in most host organs and fluids (Merhej and Raoult, 2011, Abramowicz et al., 2011). The etiologic agent of murine typhus, *R. typhi*, does not reduce the life span of flea vectors (Merhej and Raoult, 2011, Valbuena and Walker, 2009). *Rickettsia typhi*, like *R. prowazekii*, multiplies almost exclusively in the flea's gut epithelial cells and is also excreted in the feces (Merhej and

Raoult, 2011, Valbuena and Walker, 2009). Multiplication of bacilli in the salivary glands and ovaries of the arthropod host facilitates horizontal transmission during feeding and vertical TOT from adult to offspring (Merhej and Raoult, 2011, Abramowicz et al., 2011). The transmission of murine typhus to humans is usually by introduction of infected flea feces into abraded skin caused by scratching, but the oriental rat flea (*X. cheopis*), has been experimentally shown to transmit via its bite (Valbuena and Walker, 2009). Transmission also occurs when infectious flea feces or flea tissues enter the vertebrate host's respiratory tract, or conjunctivae (Abramowicz et al., 2011).

Another rickettsial species associated mainly with fleas is *R. felis*, which has been observed, through ultrastructural studies, in cat flea salivary glands (Macaluso et al., 2008, Merhej and Raoult, 2011). Unlike *R. typhi*, *R. felis* infection is credited to flea saliva instead of feces (Merhej and Raoult, 2011, Abdad et al., 2011). Stable vertical transmission of *R. felis* in cat flea populations was initially thought to occur, based on these rickettsiae being observed in flea reproductive tissues. It was consequently confirmed by PCR analyses when *R. felis* was detected in both newly laid flea eggs, indicating TOT, and in recently emerging, unfed adult fleas, indicating transstadial transmission (Azad et al., 1992, Brown and Macaluso, 2016). The *R. felis* prevalence in cat flea populations is highly variable with prevalence in a single, adult cat flea colony ranging from 35 to 96 % over the course of a single year (Reif et al., 2008, Reif and Macaluso, 2009, Brown and Macaluso, 2016). In addition to being transmitted trans-stadially and transovarially in cat fleas (*Ct. felis*), *R. felis* has been shown to be horizontally transmitted between infected and uninfected cat fleas during co-feeding on a host (Reif et al., 2008, Reif and Macaluso, 2009, Brown and Macaluso, 2016, Ehounoud et al., 2017, Angelakis et al., 2016). Despite fleas being the primary reservoir and host for *R. felis*, the bacterium has been associated with other arthropod hosts as well. Since its initial description in 1990, *R. felis* has been described in over 20 flea species, mites, lice, mosquitoes, and ticks (both Ixodid and *Argasid*), rendering terminology such as FBSF/cat-flea typhus, used to describe *R. felis*, somewhat inaccurate (Angelakis et al., 2016, Abdad et al., 2011, Socolovschi et al., 2010, Socolovschi et al., 2012a). Researchers demonstrated by quantitative PCR that *R. felis* were present in mosquitoes following experimental feeding on a bacteremic a host or bacterial suspension (Berrang-Ford et

al., 2012), and found that *R. felis* persisted in the experimentally-infected mosquitoes for up to fifteen (15) days after feeding on the infective source (Dieme et al., 2015). Despite no observable vertical transmission of *R. felis* in the mosquitoes, the bacteria were detectable in mosquito feces up to fourteen (14) days after infection, and were visible in salivary glands, in and around the gut, and in the ovaries by immunofluorescence assays. In addition, transient rickettsaemias developed in mice bitten by *R. felis*-infected *An. gambiae* mosquitoes, indicating that a malaria-transmitting mosquito species is also potentially capable of being a *R. felis* vector for human transmission (Dieme et al., 2015, Abdad et al., 2018).

Other rickettsias transmitted by insect vectors include *R. akari* generally transmitted by *Liponyssoides sanguineus* mites (Blanton, 2019, Abdad et al., 2018). Upon skin inoculation, dendritic cells phagocytose the organisms, which then travel through the lymphatic system to local lymph nodes where replication occurs. The pathogenic organisms consequently travel into the bloodstream, disseminating to infect the microcirculation endothelium, damaging the blood vessels and causing increased vascular permeability (Blanton, 2019). The result is the presentation of a cutaneous rash, and in severe cases, the manifestation of interstitial pneumonia, meningoencephalitis, acute kidney injury, multi-organ failure, and even death (Blanton, 2019, Stewart and Stewart, 2021, De Vito et al., 2020).

2.4 CLINICAL ASPECTS

2.4.1 CLINICALLY IMPORTANT RICKETTSIAS

Rickettsias are often the causative agents of severe febrile illnesses with multi-organ involvement. Clinical diagnosis of SFG rickettsiosis may be fairly uncomplicated for patients from endemic regions presenting with a clinical history of tick bite accompanied by skin rash, headache, and fever (McClain and Sexton, 2019), but the non-specific 'flu-like symptoms, i.e. fever, headache, rash, and vasculitis often associated with rickettsioses may lead to delays and complications in early detection and diagnosis (Bechah et al., 2011, Kisten et al., 2021) particularly in patients presenting in non-endemic areas. Although infections are generally mild, they may lead to death if appropriate antibiotics are not timeously administered, even in young

immunocompetent individuals (Valbuena and Walker, 2009). Patients presenting for treatment in the early stages of rickettsial infection often lack a history of a tick bite or tick contact, are without a skin rash, and present with a clinical picture suggestive of an undifferentiated viral illness, whilst others present with atypical symptoms suggestive of undifferentiated sepsis syndromes with multi-organ failure, encephalitis, myocarditis, or pneumonia (McClain and Sexton, 2019).

The primary target cells of rickettsial diseases are endothelial cells. Damaged and circulating infected endothelial cells are a clinical feature in patients infected with SFG rickettsias (Valbuena and Walker, 2009). A common indicator of rickettsial infection is localized inflammation and the appearance of an eschar or ‘tache noir’ at the site of arthropod bite; however, eschar-less and spotless rickettsioses do occur in certain cases. It is thought that the appearance of this eschar is the body’s robust reaction aimed at localized control of an infection (Abdad et al., 2018). Disseminated rickettsial infection may present with severe vasculitis and damage of the endothelial cells, clinical manifestation of skin necrosis and digital gangrene, meningoencephalitis, pneumonitis, and multi-organ failure (Abdad et al., 2018, Stewart and Stewart, 2021). When the lung and brain endothelial cells are injured, the more severe presentations of rickettsial diseases are observed, such as non-cardiogenic pulmonary oedema, adult respiratory distress syndrome, seizures, interstitial pneumonia, meningoencephalitis, and coma (Valbuena and Walker, 2009). Other serious manifestations include haemorrhagic phenomena, acute renal failure, focal neurological deficits, hypovolemic hypotension caused by leakage of intravascular fluid into the extravascular space and peripheral oedema (Valbuena and Walker, 2009, Stewart and Stewart, 2021).

2.4.1.1 Spotted Fever Group Rickettsial Infections

There are approximately fifteen (15) known spotted fever rickettsioses, including Rocky Mountain spotted fever (RMSF), boutonneuse fever, ATBF, Japanese spotted fever, Finders Island spotted fever, Queensland tick typhus, North Asian tick typhus and rickettsialpox, each caused by rickettsial species endemic to various regions globally (Mahajan, 2012). Spotted fever

group rickettsial pathogenicity is widely variable between species and despite noteworthy phenotypic variability, the majority of the SFG species are considered as potentially pathogenic to humans (Abdad et al., 2011). Generally, pathogenic species cause undifferentiated febrile illness that mimics a number of other diseases – particularly in areas where mosquito-borne illnesses and other infectious diseases are prevalent (Blanton, 2019, Stewart and Stewart, 2021). Spotted fever group rickettsiae are typically linked with tick species, albeit not exclusively so (Fournier and Raoult, 2009, Fang et al., 2017, Valbuena and Walker, 2009, Robinson et al., 2019). For example, *R. felis* is mainly associated with cat fleas (*Ct. felis*) (Abdad et al., 2011) but has also been identified in at least twelve (12) other flea species, three (3) mite species, eight (8) tick species (Bitam, 2012, Robinson et al., 2019) and at least two (2) mosquito species (Angelakis et al., 2016, Dieme et al., 2015, Berrang-Ford et al., 2012), and it is possible that *R. felis* is carried by other arthropod species too (Angelakis et al., 2016). Within the SFG are a number of highly pathogenic organisms that cause febrile illness accompanied by cutaneous rash (Figure 2.3) in those who become infected (Fournier and Raoult, 2009, Walker and Ismail, 2008). Other prominent features of SFG-related infection include headache and myalgia, localised lymphadenopathy and a typical inoculation eschar ('tache noire') at the tick bite site (Brouqui et al., 2004, Stewart and Stewart, 2021), and although not primarily a gastrointestinal illness, some patients may experience abdominal pain, nausea and vomiting – particularly in persons with severe SFG rickettsioses, such as RMSF (Blanton, 2019, Robinson et al., 2019). Clinical presentation of tick-borne rickettsioses is typically observed around six (6) to ten (10) days after a tick bite has occurred (Brouqui et al., 2004) but it can take as long as fifteen (15) days for the onset of illness (Robinson et al., 2019).

Diagnosis is usually made by serological techniques, the gold standard being IFA, whereby a four-fold rise in titre must be shown between acute and convalescent samples from a suspected spotted fever patient (De Vito et al., 2020). Diagnosis is usually guided by patient history of travel to endemic regions where a particular rickettsiosis is prevalent, the history of being bitten by a tick, flea or mite, and where possible, by the presence of a bite site eschar (Stewart and Stewart, 2021).

Spotted fever group rickettsiae grow optimally at 32°C, have a G+C content between 32% and 33%, can polymerize actin and therefore enter into host cell nuclei, cross-reacting with *Proteus vulgaris* OX-2;3,6,7 (Fournier and Raoult, 2009, Stewart and Stewart, 2021). They take from 10 to 30 days to isolate by culture (De Vito et al., 2020). Culturing requires that specimens be collected at peak of fever, be inoculated ‘fresh’, i.e. not previously frozen, and specimens should have been drawn from a patient before the commencement of antimicrobial treatment (De Vito et al., 2020). Culture isolation of SFG rickettsiae is difficult due to (1) the fastidiousness of the organisms, (2) the need for inoculation into specialized cells and cell culture media or embryonated eggs, and (3) the highly infectious nature of SFG bacteria requiring that culturing only be attempted in specialized containment (biosafety level 3) laboratories by competent professionals (De Vito et al., 2020). In the last two (2) decades, there has been a rapid development in molecular techniques for rickettsial diagnostics, and although these techniques are currently localized to larger, specialized laboratories, technologies are becoming more affordable and will hopefully be available to even the smaller satellite laboratories in due course (Broadhurst et al., 1998a, Stewart and Stewart, 2021, De Vito et al., 2020).

2.4.1.1.1 African tick bite fever

African tick-bite fever is a decidedly widespread and largely non-fatal rickettsiosis of the SFG caused by *R. africae* (Walker, 2007). The causative agent of ATBF was probably first discovered in 1930 when Adrianus Pijper isolated it, but the isolate was consequently lost (Althaus et al., 2010). In 1990, *R. africae* was isolated and later proven to be the causative agent of ATBF in 1992 (Kelly et al., 1992, Binder and Gupta, 2015, Althaus et al., 2010). Before its isolation, it was believed that ATBF was caused by *R. conorii* (Binder and Gupta, 2015, Adjou Moumouni et al., 2016). Through electron microscopy, it has been shown that the bacteria occur freely in the host's cell cytoplasm, and possesses an outer slime layer and a trilaminar cell wall (Jensenius et al., 2003a). The *R. africae* genome is comprised of one spherical chromosome (approximate size ~1248 kb) and founded on initial genotypic studies, expresses highly immunogenic lipopolysaccharide antigens, which account for extensive cross-reactivity with the other SFG rickettsiae (Jensenius et al., 2003a). High-molecular-weight rickettsial outer membrane proteins A (rOmpA) and B (rOmpB) contain species-specific protein antigens. *Rickettsia africae* can

unfortunately not be cultivated in cell-free media, although growth occurs in the yolk sacs of developing chicken embryos, and in cell cultures (Jensenius et al., 2003a). In southern Africa, *R. africae*, transmitted by *Amblyomma* ticks, mostly infects eco-travelers who visit and walk through the bushveld (Mahajan, 2012). Onset of illness occurs approximately five (5) to seven (7) days after a tick bite, much like many other TBD (Binder and Gupta, 2015, Althaus et al., 2010, Bellini et al., 2005) and is mostly considered 'spotless' although in some cases, a sparse and sometimes vesicular rash may be observed (Figure 2.3) (Mahajan, 2012, Adjou Moumouni et al., 2016). ATBF varies from the majority of other rickettsioses of mild-to-moderate severity in certain physiological aspects and expresses as acute, febrile, and flu-like illness, commonly accompanied by intense headaches, noticeable neck muscle myalgia, 'tache noire' inoculation eschars, with distinctive red halo surrounding a black crusty center at the tick bite site, and painful localized lymphadenitis (Lepidi et al., 2006, Binder and Gupta, 2015, Walker, 2007, Althaus et al., 2010). The formation of an eschar at the site of the tick bite is a result of rickettsial proliferation in endothelial cells. Due to the aggressive biting habits of the *Amblyomma hebraeum* (Figure 2.4) and *A. variegatum* ticks, multiple eschars are often observed in ATBF cases (Walker, 2007).

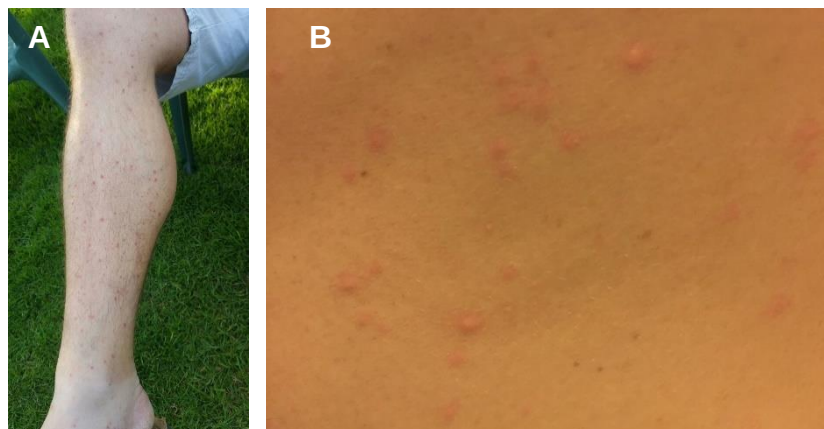


Figure 2.3. Patient with mild tick bite fever. A: right leg with cutaneous rash; B: skin eruption following visit to tick infested area. Photo: Dr J Weyer.



Figure 2.4. Adult *Amblyomma hebraeum* male tick. Primary vector of *Rickettsia africae*, the causative agent of African tick bite fever. Photo: Alan Kemp & Anastasia Trataris-Rebisz

Diagnosis is often troublesome due to the presentation of a wide spectrum of non-specific symptoms similar to any number of tropical, febrile diseases of unknown origin (Adjou Moumouni et al., 2016). In addition, immunoglobulin M (IgM) and IgG are both usually only detectable around seven (7) to fifteen (15) days following disease onset in most tick-borne rickettsioses, but not necessarily in ATBF patients. The IgG and the IgM responses to *R. africae* are usually delayed, with seroconversion only observed at 28 and 25 days after the onset of symptoms for IgG and IgM, respectively (Brouqui et al., 2004). For obvious reasons, this complicates confirmation of the diagnosis. Epidemiological information, such as potential tick vectors and/or SFG rickettsiae in a specific area, therefore prove valuable for diagnosing a suspected ATBF case, and helps in determining the control and prevention strategies (Adjou Moumouni et al., 2016).

In Chapter 5 of this dissertation, ATBF is brought into focus as a potential differential diagnosis for patients presenting with serious conditions, in particular, patients thought to have been infected with Crimean-Congo haemorrhagic fever (CCHF) virus.

2.4.1.1.2 Flea-borne spotted fever

Rickettsia felis, an emerging insect-borne rickettsial pathogen, is the responsible cause of FBSF (Brown and Macaluso, 2016). Since its initial description in 1990, *R. felis* has been reported globally and is considered a typical cause of febrile illness in Africa (Brown and Macaluso, 2016, Ehounoud et al., 2017). In sub-Saharan Africa, recent studies have reported that up to 15% of patients with 'fever of unknown origin' have been found to be infected with *R. felis* and there is evidence to suggest that this organism is probably ubiquitous, with *R. felis* infections probably being misdiagnosed as other febrile illnesses (Mediannikov et al., 2013, Angelakis et al., 2016). The widespread distribution of *R. felis* has been accredited to the global dissemination of cat fleas (*Ct. felis*) its principal vector and reservoir (Brown and Macaluso, 2016). In 2002, the *R. felis* reference strain (Marseille-URRWXCal2) was cultured from cat fleas by culturing in an XTC-2 cell line incubated at temperatures < 32°C (Brown and Macaluso, 2016, Angelakis et al., 2016). Reports of *R. felis* as the infectious agent of FBSF drastically escalated following the isolation of the reference strain (Brown and Macaluso, 2016). Genetic analyses of *R. felis* has revealed a large genome size and the presence of plasmids when compared with other rickettsiae (Brown and Macaluso, 2016). *Rickettsia felis* was originally categorized with SFG rickettsias due to the incidence and incomplete expression of the *ompA* gene, essential for rickettsial taxonomy, but it was later proposed it should be classified in a discrete 'transitional' group, on account of its conjugative plasmid (Angelakis et al., 2016). The validity of the transitional group is however contested and members of this group are often considered part of the SFG.

Due to non-specificity of the clinical signs (e.g., headache, fever, rash, and myalgia), FBSF cases are likely an underestimated emergent global human health hazard. In addition, inadequate access to suitable laboratory tests such as molecular diagnostics, may compound this problem (Abdad et al., 2011, Brown and Macaluso, 2016). It was reported that the earliest human case of *R. felis* infection was incorrectly diagnosed as flea-borne endemic typhus caused by *R. typhi*, the failure attributed to the limited capacity of accessible serological assays to differentiate between the two (2) rickettsial species (Brown and Macaluso, 2016). Molecular studies are also problematic for *R. felis* owing to inconsistent tree topologies rife with

uncertainties that have recently been demonstrated from single- and multigene analyses (Angelakis et al., 2016).

2.4.1.1.3 Mediterranean spotted fever

Rickettsia conorii, the causative agent of MSF, is the cause of a significant number of deaths worldwide. The disease caused by *R. conorii* has been known by various names depending on the geographic area where it occurs, including South African tick typhus, Indian tick typhus, Boutonneuse fever, Marseilles fever, or even escaro-nodular fever (Brouqui et al., 2004, Blanco and Oteo, 2006, Broadhurst et al., 1998a). It was thought to be the sole rickettsiosis prevailing in Europe until the early 21st century. The etiologic agent, *R. conorii*, is mainly spread by the brown dog tick (*Rhipicephalus sanguineus*) (Brouqui et al., 2004, Blanco and Oteo, 2006), but has also been isolated from various other Ixodidae ticks, i.e. *Rhipicephalus*, *Hyalomma*, and *Haemaphysalis* species (Blanco and Oteo, 2006, Bitam, 2012). *Rickettsia conorii* was first isolated in Tunisia in 1932 (Bitam, 2012) and has since been isolated and reported to cause significant morbidity globally (Broadhurst et al., 1998, Cowan, 2000, Walker, 2007, Walker and Ismail, 2008). Case numbers increase throughout the warmer seasons (end of spring and summer) and the disease incubates for around seven (7) days before the abrupt onset of MSF illness. Patients present with elevated fever, headache, malaise, rash typically on palms of hands and soles of feet and usually a blackened eschar at the tick bite site (Blanco and Oteo, 2006). *Rickettsia conorii* infections have also been reported to present with a clinical picture mimicking that of viral hemorrhagic fevers (de Almeida et al., 2010). Due to the limited capacities of the laboratories in many of the areas where *R. conorii* is present, disease diagnosis is often unsupported by laboratory results, which results in suspected clinical diseases being unconfirmed and appropriate treatment timeously administered, lacking (Broadhurst et al., 1998). Indirect fluorescent antibody tests and other sophisticated procedures are usually only available in urban central laboratories with access to specialized equipment and highly skilled personnel, creating a diagnostic dilemma for clinical practitioners treating indigenous patients as well as visitors to endemic areas (Broadhurst et al., 1998).

2.4.1.2 Typhus Group Rickettsial Infections

'Typhus', a term originally used in the 1760s, describes the typhus disease complex, which is comprised of a group of related communicable agents that cause epidemic typhus, murine typhus and previously, scrub typhus (Fuxelius et al., 2007). The TG rickettsiae are transmitted only by insects, i.e. species transmitted by fleas (*R. typhi*), and lice (*R. prowazekii*) (Valbuena and Walker, 2009). They grow optimally at 35°C, have a G+C content of 29%, are incapable of polymerizing actin or entering host cells' nuclei (i.e. are only found in the cytoplasm) and cross-reaction occurs with *Proteus vulgaris* OX-196,7 (Plotz et al., 1943, Emelyanov, 2001, Fournier and Raoult, 2009).

Typhus group rickettsiae are often transmitted without the patient being aware of exposure, which can occur in a rodent-infested environments like ports, markets, and city centers or urban settlements with poor sanitation and sub-par pest control (Walker, 2003, Blanton, 2019). Symptoms of disease present as elevated fever, headache and diverse neurological signs, and before the antibiotic era, death rates may well have been as high as 40% in diseased patients (Walker, 2003, Blanton, 2019). *Rickettsia tsutsugamushi*, an agent vectored by mites and causative agent of scrub typhus, was previously included in the TG, but with advancements in molecular phylogenetic analysis, the species was renamed *Orientia tsutsugamushi* and removed from the group (Parola and Raoult, 2006, Perlman et al., 2006, Hawley et al., 2007).

2.4.1.2.1 Epidemic typhus

Also known as Brill-Zinsser disease, classic typhus, typhus exanthematicus (French), tarbadillo (Spain), Fleckfieber (German) and jail fever

'Typhus' is derived from the archaic Greek word for 'fever with stupor' - τυφος. The principal disease and recrudescent form (Brill-Zinsser disease) are caused by *R. prowazekii* (Mahajan, 2012, Blanco and Oteo, 2006). Epidemic typhus (mortality rate 10 to 60%) clinically resembles murine typhus (mortality rate < 2%) (see 2.4.1.2.2. below), albeit more severe, and presents with serious neurological manifestations such as confusion, delirium, deafness, and even coma and can manifest in a recurrent form, years after the initial infection. The recurrent form of epidemic typhus may cause new epidemics if it arises at a time and place of diminished living and hygiene

conditions favoring the increased incidence of the human body lice (Fuxelius et al., 2007, Valbuena and Walker, 2009, Fang et al., 2017). This disease shares a close association with war, poverty, natural disasters, and famine (Blanco and Oteo, 2006). Infection is characterized by the early onset of malaise followed by sudden fever, headache, chills, and myalgia. A macular rash usually starts in the axilla or on the trunk and extends to the extremities (Fang et al., 2017). Early descriptions of typhus usually reported the presence of a rash, and considered this symptom a diagnostic criterion for the disease. In recent outbreaks only 25% to 38% of patients were noted to present with a rash, although recent outbreak reports come from Africa, where rash may have been missed in a darkly pigmented population (Fang et al., 2017). As is true for other rickettsiae, *R. prowazekii* causes vasculitis when the endothelial cells of capillaries, smaller arteries and veins are infected, leading to blood vessel occlusion by fibrin and platelet deposition (Mahajan, 2012). TG rickettsiae extensively proliferate, accumulating intracellularly until the endothelial cells burst and disseminate the pathogen through the bloodstream (Mahajan, 2012). Recrudescence typhus presentation often resembles non-fatal primary infection (Fang et al., 2017). The infection often self-resolves, but the bacterial infection is capable of persisting indefinitely for the duration of the human host's lifespan, and resurface as Brill–Zinsser disease under stress conditions such as poor living conditions, lapses in public health coverage and support, natural disasters, war, and so on (Blanco and Oteo, 2006). An outbreak of epidemic typhus can occur when body louse infestation is rampant in a population since the clinical form of the disease is bacteraemic (Blanco and Oteo, 2006).

2.4.1.2.2 Murine Typhus

Murine typhus, also known as flea-borne, rat, urban and endemic typhus, caused by *R. typhi* (formerly *Rickettsia mooseri*), is classically associated with Norwegian rats (*Rattus norvegicus*), roof rats (*Rattus rattus*), and the Oriental rat flea (*X. cheopis*) (Abramowicz et al., 2011, Bitam et al., 2010). Murine typhus was first described in the early 20th century in the United States where it was being widely diagnosed and reported. In the years that followed, incidence showed a sharp decline as a result of the wide urban implementation of rodent and flea control efforts (Abramowicz et al., 2011). In the last two (2) decades, numerous reports have stressed the importance of this re-emerging disease in travelers returning from endemic regions (Bitam et al.,

2010). The incubation periods range from 6-14 days and the majority of the symptoms are common to various other infectious diseases; thus, cases are often missed or misdiagnosed (Bitam et al., 2010). Infected human hosts usually present with elevated fever, headache, weakness, generalized pain, and several other non-specific indicators or atypical presentations (Abramowicz et al., 2011). *Rickettsia typhi* generally infects flea mid-gut epithelial cells and mammalian hosts' endothelial cells, and is excreted in flea feces. Human transmission occurs through flea fecal contamination introduced into skin abrasions during scratching of flea bites, inhalation of the dried feces or contamination of the conjunctiva with infective flea feces (Bitam et al., 2010, Blanco and Oteo, 2006). *Rickettsia typhi* is sometimes difficult to consider as the cause of an infection due to the absence of rats and their fleas. Diagnoses of human cases are usually made by serological techniques, since culture is difficult and impractical, which are often complicated by the cross-reactivity between rickettsial species. (Blanco and Oteo, 2006). To further complicate diagnosis, *R. felis* also presents as a murine typhus-like rickettsiosis, despite being a SFG rickettsial species transmitted by cat fleas (Blanco and Oteo, 2006).

2.4.2 MEDICAL MANAGEMENT, DIAGNOSIS AND TREATMENT

Despite the accessibility of relatively affordable and effective antibacterial therapy, tick-borne rickettsial diseases continue to cause notable morbidity and mortality in healthy persons (Biggs et al., 2016). It is important to recognize rickettsioses early in their clinical progression since antibacterial therapeutics are most effective if initiated early. This is, however, not always possible since the early signs and symptoms of rickettsial infections are often nondescript and tend to resemble other illnesses, thus complicating diagnosis (Biggs et al., 2016, Stewart and Stewart, 2021). Rickettsial infections are commonly missed or misdiagnosed, principally due to a deficiency of diagnostic consideration and suitable laboratory testing (Walker, 2003, Stewart and Stewart, 2021). Rickettsioses are notoriously perplexing to diagnose, and, with the absence of rapid, point-of-care tests available for acute infection diagnosis, disease confirmation is usually by serological assays of retrospective specimens (Blanton, 2019). Disease usually presents as acute non-specific febrile disease associated with symptoms including nausea, vomiting, myalgia, and headaches (Walker, 2003, Stewart and Stewart, 2021). In early onset of disease, i.e. the first three (3) or so days, there are several differential diagnoses that share

endemicity with rickettsioses that are often considered, including bacterial, protozoal, and viral enterocolitis, malaria, dengue fever, hepatitis and other arboviral infections, typhoid fever, enteroviral infection, leptospirosis, infectious mononucleosis, and urinary tract infections (Walker, 2003). It is prudent to consider rickettsioses as potential etiologic agents of the non-specific febrile illness when performing diagnostic work-up of a patient with recent travel history to endemic areas, especially when there is an eschar and history of tick- or flea-bites (Walker, 2003, Stewart and Stewart, 2021). There are a number of infections associated with the appearance of localized cutaneous ulcer/s (eschars) accompanied by undifferentiated fever, including ATBF, rickettsialpox, Boutonneuse fever, Queensland tick typhus, scrub typhus, North Asian tick typhus, Flinders Island spotted fever and Japanese spotted fever (Walker, 2003).

Tick-borne SFG rickettsioses diagnoses were formerly confirmed almost entirely by serological techniques, until the development and usage of cell culture systems and molecular techniques (Brouqui et al., 2004, Parola et al., 2013, Schjørring et al., 2020). Even a positive microimmunofluorescence (MIF) assay, the current reference method, cannot definitively confirm that the patient's disease was caused by a particular rickettsial species, due to antigenic cross-reactivity among SFG rickettsiae (Brouqui et al., 2004, Parola et al., 2013). There are a number of diagnostic methodologies available for the detection of rickettsial infections, ranging from serological techniques including IFA, ELISA (Blanda et al., 2017), and immunochromatographic assays, to various molecular techniques such as real time PCR, and multiplex PCRs (Schjørring et al., 2020, Stewart and Stewart, 2021, Abdad et al., 2018, De Vito et al., 2020).

Serologic tests are the most extensively available laboratory diagnostic technique relying on the principles of antigen-antibody binding (Koivunen and Krogsrud, 2006, Fritz, 2009). These assays offer straightforward, swift, robust yet sensitive, and straightforwardly automated methods, pertinent to routine testing in medical laboratories (De Vito et al., 2020). Most methods are based on simple photo-, fluoro-, or luminometric detection. Antibodies associated with specific diseases, disease biomarkers, hormones, and pharmaceuticals are quickly detected and results are generally easy to interpret (Koivunen and Krogsrud, 2006). Upon review of all commercially-

available diagnostic offerings for rickettsial infections, the two (2) methodologies most often used are ELISA and IFA. Enzyme-linked immunosorbent assays offer rapid, sensitive, easy and non-hazardous detection of either antigens or antibodies in clinical samples and is widely preferred in low-resource settings owing to the ability for batch testing and decreased requisite for high technical expertise (De Vito et al., 2020, Stewart and Stewart, 2021). Secondary antibodies are conjugated with enzyme molecules that bind to the primary antigen-antibody complex. Upon addition of an appropriate substrate, the enzyme catalyzes the production of a coloured end-product, and it is this coloured end-product that is visualized and quantified. Separation of specific antigens from nonspecific complexes is achieved through binding of the antigen or capture antibody to a solid matrix such as polystyrene microtitre plate, and through repeated washings nonspecific binding is minimized (Koivunen and Krogsrud, 2006). There are two types of immunofluorescence assays, direct fluorescent antibody test (DFA) used for histochemistry, and IFA test. For DFAs, the antigen-specific fluorophore-labelled antibody is applied to a fixed smear on a microscope slide, incubated, washed, and visualized under a fluorescence microscope, whereas an IFA differs in that it is the secondary, species-specific antibody, instead of the primary antibody, is labelled with a fluorophore, e.g. FITC, Texas Red. In both IFAs and DFAs, specific monoclonal or polyclonal antibodies are conjugated to fluorescent dyes (fluorochromes), which can be visualized using a fluorescence microscope when the fluorophores are excited by light at a specific wavelength, and they release the extra energy by emitting light at another, longer wavelength (Koivunen and Krogsrud, 2006).

Another commonly utilized serological technique is the WF test, although this test method has over the years fallen out of use, particularly in urban laboratories with improved technologies available (De Vito et al., 2020). This assay is established on the recognition of antibodies to various *Proteus* species containing antigens with cross-reactive epitopes to antigens of *Rickettsia* species (except *R. akari*). Serum collected from a patient infected with a SFG rickettsiae (except RMSF) will react strongly with *P. vulgaris* OX-2 whole cells, and *P. vulgaris* OX-19 whole cells react with sera from persons infected with TG rickettsiae and RMSF. Sera from scrub typhus patients agglutinate with *Proteus mirabilis* OX-K strain and thus assist with the diagnosis of *O. tsutsugamushi*- related infections (Stewart and Stewart, 2021).

Culture isolation is crucial when investigating virulence, antimicrobial susceptibility, and whole-genome sequencing of pathogens (Lagier et al., 2015, Stewart and Stewart, 2021); however, it has long been accepted that the Rickettsiae do not easily grow in any rich artificial medium. It has been increasingly shown that tick cell lines play an essential role for research on tick-borne pathogens (TBPs) and symbionts like *Rickettsia* spp., whereby isolation of fastidious tick-borne microorganisms has been aided by primary tick tissue or organ cultures, tick cell lines, or combinations of two or more systems (Alberdi et al., 2012, Bell-Sakyi et al., 2007). Reports published as early as 1964 have shown the value of these tick cell lines over chick embryo cell culture for the isolation of minute quantities of tick-borne encephalitis virus (Alberdi et al., 2012, Rehacek and Kozuch, 1964). Ultimately, the goal for pathogen diagnosis is isolation of the microbial agent from an arthropod or patient (Brouqui et al., 2004). *Rickettsia* species are most frequently isolated from blood (decanted plasma collected in heparin or citrate anticoagulant tubes), cutaneous biopsies (particularly the inoculation eschar) or tick hemolymph inoculated into tissue cell cultures (Brouqui et al., 2004). It is recommended that isolation attempts be carried out in high containment facilities (e.g. biosafety level 3 laboratories) due to the infectious nature of these bacteria. Cultured bacteria are generally demonstrable 48–72 h post-inoculation (Brouqui et al., 2004) but have been known to take as long as 10 to 30 days (De Vito et al., 2020). Microscopic investigation after Gimenez staining, immunodetection or PCR support in the discovery and confirmation of rickettsiae within the cell culture cells. If after 20 days, immunofluorescence remains negative, the culture is considered negative. Parallel shell vials are inoculated on to confluent monolayers, if immunofluorescence is determinately positive. This is done using tissue culture flasks containing human erythroleukemia cell lines, in an attempt cultivate rickettsial isolates (Brouqui et al., 2004).

Over two (2) decades ago, the focus of rickettsial diagnostics shifted from serological typing and identification to molecular approaches (De Vito et al., 2020, Robinson et al., 2019). This technique allows for detection of pathogen DNA far sooner during the course of infection than serological techniques that typically require seroconversion shown in convalescent samples taken up to three (3) weeks following the onset of illness; by which time the antibiotic treatment

will have already been started (Stewart and Stewart, 2021). Molecular testing requires a specimen, i.e. eschar biopsies, blood buffy coats, swabs of vesicular lesions etc., to be extracted and tested using pathogen-specific primers (Bechah et al., 2011). This technique is both sensitive and specific and PCR products can be sequenced to determine the infecting species. A 'downside' to this method is specimen collection, particularly for eschar biopsies, which is invasive, sometimes quite agonizing for patients and challenging to obtain for certain parts of the body. Rapid, noninvasive, and painless techniques are always preferable and beneficial for the successful diagnosis of rickettsioses in patients (Bechah et al., 2011). Advances in the analyses and comparisons of *gltA* sequences of various *Rickettsia* species aided the molecular diagnostic approach (Guillemi et al., 2015, Roux et al., 1997). It was discovered that the average rate of sequence change in *gltA* was greater than in 16S rRNA. This indicated that *gltA* was less conserved in rickettsiae, a trait that was revealed valuable in discriminating between *Rickettsia* species (Guillemi et al., 2015). PCR-based molecular methods have facilitated the advance of sensitive, specific and rapid tools for detection and identification of *Rickettsia* species in specimens such as blood, cutaneous biopsies (i.e. 'tache noire') and even ticks and other ectoparasites (Brouqui et al., 2004). Various rickettsial genes have been targeted with a range of published primer sets that may be utilized in a suitable laboratory setting (Brouqui et al., 2004).

Challenges in early diagnosis, particularly since early presentation can resemble viral infections, often lead to the incorrect treatment regimens being prescribed for patients with a rickettsial infection (Talikoti and Deka, 2019). The empirical drug of choice for treatment of rickettsial infections is doxycycline (Frean et al., 2008), with azithromycin recommended for pregnant women since doxycycline is contraindicated in this group (Talikoti and Deka, 2019, Frean and Grayson, 2019). It is generally recommended for adults, that 100 mg doxycycline be taken twice daily for five (5) to seven (7) days or for 48 h after resolution of fever (Althaus et al., 2010, Frean and Grayson, 2019). The possibility of different diagnosis should be considered if a patient suspected of having a SFG rickettsiosis does not demonstrate any clinical response within 48 h since doxycycline is highly effective against *Rickettsia* infections (Frean and Grayson, 2019). In cases where critically ill patients cannot tolerate oral medication, a fluoroquinolone like ciprofloxacin may be the sole treatment option available, since it is safer than chloramphenicol,

the drug of choice for these situations in the past. This is especially true if parenteral tetracycline is unavailable, as is the case in South Africa (Frean and Grayson, 2019, Frean et al., 2008). Erythromycin has been reported to have poor efficacy, with limited clinical data recommending new macrolides like clarithromycin and azithromycin, but may be used to supplement the initial doxycycline treatment to cover other possible bacterial pathogens (Frean and Grayson, 2019). Doxycycline should be considered for all patients presenting with symptoms other than mild since severe SFG rickettsioses can be fatal in patients of any age group. In this situation it is recommended that a one (1) to two (2) day course of doxycycline be followed by three (3) to five (5) day course of a macrolide, in children younger than eight (8) years of age and pregnant women (Frean and Grayson, 2019).

CHAPTER 3 - COMPARISON OF COMMERCIAL ASSAYS FOR THE SEROLOGICAL INVESTIGATION OF SPOTTED FEVER RICKETTSIOSIS

3.1 INTRODUCTION

Although molecular methods are increasingly being used, serology remains the most widely used approach for the laboratory investigation of rickettsiosis. The first serological test was developed in 1916 by co-workers Weil and Felix (Eldin et al., 2019, Kantsø et al., 2009, Raoult and Roux, 1997) and detects SFG rickettsial antibodies (except *R. rickettsia*), if present in the patient's serum through the visualisation of SFG antibody agglutination when presented with *Proteus vulgaris* OX2, *P. vulgaris* OX19 and *Proteus mirabilis* OXK antigens (Kantsø et al., 2009, Raoult and Roux, 1997). The test is inexpensive and easy to perform, requiring only basic laboratory equipment, therefore despite its low sensitivity and specificity, is still routinely used in developing countries like India where resources are limited and alternative methods are not readily available (Kularatne and Gawarammana, 2009, Eldin et al., 2019, Srinivasagam et al., 2018, Sanap et al., 2017). In general, serological techniques remain the most viable option for many laboratories conducting *Rickettsia* diagnostics for several reasons; 1) serological assays do not rely on the pathogen being present in the sample being tested, 2) can be done using basic laboratory techniques and technologies and generally does not require expensive specialized equipment such as thermocyclers, and 3) testing is relatively inexpensive per sample tested as a large number of samples can be batched and tested together (Brouqui et al., 2004, Raoult and Roux, 1997, Portillo et al., 2017). The IFA is still considered the gold standard for serological diagnosis of rickettsial infections (Kantsø et al., 2009, Fomda et al., 2021). Although a single reactive IFA titre in a patient presenting with symptoms and clinical history suggestive of the diagnosis may be valuable, confirmation of a *Rickettsia* infection requires demonstrable seroconversion, or a four-fold rise in titre between acute-and convalescent-phase sera (Blanton, 2019). It is also important to note, group-specific antigens are generally cross reactive, so serology is incapable of offering a species-specific diagnosis devoid of the need to perform cumbersome cross absorption techniques, such as the Western blotting (WB) technique, in a complimentary manner to the IFAs. These cross

absorption techniques are however, often commercially unavailable, therefore this type of testing is limited to reference laboratories (Blanton, 2019). A myriad of serological testing platforms have been developed over the years (Kantsø et al., 2009), including latex agglutination, CF, ELISAs, WBs and IFAs (La Scola and Raoult, 1997, Mahajan, 2012, Sood et al., 2013, Bizzini et al., 2015, Binder et al., 2019). The advantages and disadvantages of the various assays are summarized in Table 3.0 in the supplementary materials section Appendix 3.1.

In 2015, Bizzini and co-workers reported on the development of an automated epifluorescence assay, a multiplex antigen microarray that uses standardized quantities of antigens, spotted on glass slides with appropriate controls (Bizzini et al., 2015). The CF assay was adapted to detect rickettsia-specific antibodies and results are varied depending on the how the antigen was produced and the amount of antigen used in the assay (La Scola and Raoult, 1997, Bizzini et al., 2015). Enzyme-linked immunosorbent assays were initially developed to detect *R. typhi* and *R. prowazekii* antibodies and were then later adapted to the diagnosis of RMSF and scrub typhus. This ELISA technique is highly sensitive and reproducible, and aids the differentiation between IgG and IgM antibodies (La Scola and Raoult, 1997). Despite the availability of several tests for detection of rickettsioses, the majority are inadvisable, the exceptions being IFA, WB, and cross-adsorption assay (La Scola and Raoult, 1997, Jensenius et al., 2004b). Immunofluorescence assays are able to detect IgG and IgM antibodies, with variable sensitivity rates (ranging from 42 to 100%) (Stephen et al., 2018). A major disadvantage of IFAs is the poor species specificity which may be avoided by using a multiple-antigen IFA that allows for the direct comparison of reactions to several species (Jensenius et al., 2004b). The complexity of diagnosis of rickettsial infections is primarily due to the cross-reactivity common amongst SFG *Rickettsia* (Bizzini et al., 2015). An additional limitation of serological diagnosis of rickettsias, is delayed detectable antibody response (Fang et al., 2017). Serological methods are based on the indirect detection of *Rickettsia*-specific antibodies, which characteristically are retrospectively detectable, since these antibodies are produced late in infection – long after the onset of symptoms and are thus of limited clinical impact (Papp et al., 2017). Failure to test samples taken at appropriate stages in the progression of the disease, creates the

considerable risk of misinterpretation of the data generated by serological assays, especially when no additional laboratory methods are used in the patient's diagnostic evaluation (Paddock, 2013).

Since serological techniques only detect an antibody response to infection at the earliest seven (7) to ten (10) days after onset of illness, it has been hypothesized that when tetracycline-class drugs are administered in early treatment, the development of antibodies can become diminished or delayed (Biggs et al., 2016). Therefore, alternative methods to investigate acute stages of illness are essential. A single raised antibody titre is insufficient when trying to confirm an acute rickettsial infection (Biggs et al., 2016). That being said, there is still value in serological testing, particularly in mild or moderately ill patients; when paired sera, taken at the optimal points in disease progression, are tested and a four-fold rise in serum antibodies is observed, these serological results provide the best evidence for a current infection (McQuiston et al., 2014).

Culture is undeniably the reference standard for all microbiological diagnoses, with the least amount of bias when compared with other diagnostic assays (Paddock, 2013). Culture techniques are not easily applicable or rather executable in conventional laboratories due to the nature of this pathogen. *Rickettsia* are obligate, intracellular organisms that require cell lines, embryonated chicken eggs and laboratory animals for the culture to be successfully performed (Luce-Fedrow et al., 2015, Blanton, 2019). In addition, the culture of pathogenic rickettsiae is restricted to biosafety level 3 (BSL-3) facilities and in order to culture isolates in cell culture, egg cultivation and lab animal passage, operators are required to have specialized knowledge and expertise (Luce-Fedrow et al., 2015, Cortenraad, 2014). The applicability of culture in a diagnostic capacity, is limited due to the fastidiousness of rickettsiae. Whilst some rickettsial species may be detectable as early as two (2) to three (3) days after primary inoculation in certain culture systems, the median time reported for other species is may be as long as 27 days, which is both laborious and impractical for clinical diagnosis (Luce-Fedrow et al., 2015). Due to the difficulty experienced when attempting to culture and the requirement for

specialised laboratories, the recommendation is to only attempt culture in severe cases where the species diagnosis is of utmost importance (Cortenraad, 2014, Jensenius et al., 2004a).

Several PCR-based detection methods have been developed, and are available at larger centralized diagnostic laboratories (Bizzini et al., 2015). PCR-based methods have several advantages and disadvantages. Rapid diagnosis is critical in an acute infection, but as previously mentioned, serological titres are often negative during acute phases of infection, due to the delayed development of antibodies and the prolonged persistence of these antibodies after the resolution of the infection (Kidd et al., 2008). PCR is distinctly advantageous over serology for the detection of 'active' infection from a single sample at a single time-point (Maggi et al., 2014). During the acute phase of infection *Rickettsia* species circulate in low numbers in the blood (Parola et al., 2005, Kidd et al., 2008). PCR is useful in identifying infections in this acute phase, prior to seroconversion, and is therefore effective in distinguishing between a previous exposure (i.e. where no bacteria are detectable in seropositive individuals) and an active infection (Kidd et al., 2008). In addition, sensitive PCRs can detect even a very low level of circulating bacteria, but there exists a minimum detectable level whereby circulating bacteraemia is below minimum detectable levels – in these instances, the infection is potentially missed by the PCR (Maggi et al., 2014). Specific pathogens can be targeted at species or strain level through targeted PCR primer sets or by sequencing of amplicons/PCR products (Maggi et al., 2014). An additional benefit of molecular techniques is that multiple pathogens or species can simultaneously be detected using a multiplex PCR assay. A disadvantage is that setting up and optimising these multiplex assays are potentially challenging, particularly when trying to achieve optimal sensitivity for all targeted pathogens, since co-infecting pathogens may compete in the PCR reaction process (Maggi et al., 2014). Another disadvantage of the PCR method is testing the incorrect sample type, i.e. testing specimens where *Rickettsias* are not likely to reside, or testing specimens taken after antibiotic treatment has already been initiated and thereby reduced the circulating bacteria to below minimum detectable levels (Maggi et al., 2014). This methodology is also only available in reference centres as it requires specialized equipment and expertise to run and interpret assay results.

Given that serological techniques are, by in large, the most readily available methodology for *Rickettsia* diagnosis in testing laboratories in South Africa, the aim of this study was to compare commercially-available serological diagnostic assays easily attainable on the South African market, to determine comparability between test results obtained using the various serological kits.

3.2 MATERIALS AND METHODS

3.2.1 SAMPLE SELECTION

Testing panels were compiled from residual specimens previously collected for research or surveillance programs at the National Institute for Communicable Diseases (NICD), Center for Emerging Zoonotic and Parasitic Diseases (CEZPD). Sixty-two (62) samples were selected from an acute febrile illness (AFI) of unknown etiology study (Simpson et al., 2018) and 78 samples were selected from the residual material of clinically 'well' study participants who are occupationally at risk of TBD (Msimang et al., 2014). The AFI study samples were collected from adult patients (≥ 18 years old) presenting at three rural, government primary health care community clinics (i.e. Waverdiend, Utah and Gottenburg) in Bushbuckridge Municipality, Mpumalanga Province, South Africa for treatment of acute febrile illness of unknown origin. Samples of sufficient volume to be used for several testing treatments were selected, favoring samples with both acute and convalescent samples available with consistent results. The majority of the samples selected were previously found to be negative (49/62) based on the IFA results. The clinically 'well' samples were collected from Kruger National Park (KNP) employees (including veterinarians and game rangers), working at the human-wildlife interface. Due to participants not displaying symptoms of acute illness at the time of sample collection, serological assays previously conducted, were targeted to illustrating past exposure (IgG only) rather than current infection. Of 78 samples selected, 17 were IgG negative samples and 61 were IgG positive samples. Samples were collected, processed and stored as previously reported (Msimang et al., 2014, Simpson et al., 2018). In both studies, *R. conorii* IgG and IgM IFAs (Viracell, Spain) were performed in accordance with manufacturer protocols.

3.2.2 ETHICS STATEMENT

Ethics Committee clearance for the use of the archived material was obtained prior to the study (University of Witwatersrand Human Research Ethics Committee, M120667 (amended) and the University of Pretoria Human Research Ethics Committee, 222/2017) (see supplementary materials – Appendices 3.2 and 3.3).

3.2.3 COMMERCIAL ASSAY SELECTION

An online search using the Google search engine was performed, using the following keywords: *Rickettsia*, serology, and commercial kits; to determine what commercially-produced serological assays for *Rickettsia* diagnosis were available globally (Table 3.1). Local distributors were then approached to assess the availability of these commercial kits in South Africa. Kits available in South Africa are indicated in Table 3.1 and selection of kits included in this study was based on availability within country at the time of the study.

Available kits inserts were compared and kit features were summarized (Table 3.2) for ease of comparison. The Vircell IFA is the kit that was used in the previous studies from which the panels were derived, so panel results were compared with those of the previous studies’.

Table 3.1. Commercially available serological kits for the diagnosis of Rickettsioses.

Kit manufacturer	Techniques used	Targets
Fuller Laboratories	IFA*	IgG/IgM – <i>R. conorii</i>
	ELISA*	IgG/IgM – SFG rickettsiae
Focus Diagnostics	IFA*	IgG - <i>R. conorii</i>
Vircell microbiologists	ELISA*	IgG/IgM - <i>R. conorii</i>
	IFA*	IgG - <i>R. conorii</i>
		IgM - <i>R. conorii</i>
	Chemiluminescence	IgG - <i>R. conorii</i>
		IgM - <i>R. conorii</i>
Immunospec Corporation	Immunochromato-graphic assay	<i>Rickettsia</i> spp.
DRG Diagnostics	ELISA	IgM - <i>R. conorii</i>
Gentaur	Antigen MIF	IgM - <i>Rickettsia</i> spp.
GenBio	ELISA dot	IgM/IgG - <i>R. conorii</i>
Xiamen Boson Biotech	Rapid test	IgG/IgM – <i>Rickettsia</i> spp.
SD Bioline Tsutsugamishi	Rapid test	IgG/IgM/IgA – <i>O. tsutsugamishi</i>

* Commercially available in South Africa

Table 3.2. Summary of commercial kits available for *Rickettsia* serology in South Africa.

Manufacturer	Focus Diagnostics	Fuller Laboratories	Vircell Microbiologists	Vircell Microbiologists
Target antigens	Spotted fever group (<i>R. rickettsia</i>) and Typhus group <i>Rickettsia</i>	Spotted fever group <i>Rickettsia</i> OMP antigen <i>R. conorii</i>	<i>R. conorii</i>	<i>R. conorii</i>
Target antibodies	IgG and IgM	IgG and IgM	IgG and IgM	IgG and IgM
Type of test	IFA	ELISA	ELISA	IFA
Kit Components (included in kit)	- Substrate slide (2 antigen spots) - IgG conjugate - Positive controls at screening dilution - Negative control at screening dilution - Yolk sac diluent - Mounting medium - PBS powder	- EIA microwells - IgM serum prep - Sample diluent - Enzyme conjugate - Positive control - Calibrator - Negative control - TMB substrate - Stop solution - PBS buffer - Tween 20	- EIA microwells - Sample diluent - IgG or IgM conjugate - IgG/IgM Positive controls - IgG/IgM Cut-off controls - IgG/IgM Negative controls - Stop solution - Wash buffer	<i>R. conorii</i> slide - Vircell PBS - Vircell <i>Rickettsia</i> IgG/IgM Positive Control - Vircell <i>Rickettsia</i> Negative Control - Vircell anti-human IgG FITC conjugate - Vircell mounting medium
Required but not supplied with Kit	- Micropipettes - Incubator - Distilled water - Dilution microtubes 24x60 mm coverslips - Fluorescence (UV) microscope - Humid chamber	- Micropipettes - Incubator - Distilled water - Dilution plate	- Micropipettes - Incubator - Distilled water - Dilution plate (For IgM) IgG Immunosorbant	- Micropipettes - Incubator - Distilled water - Dilution microtubes 24x60 mm coverslips - Fluorescence (UV) microscope - Humid chamber
Specificity / Sensitivity	Group sensitivity / specificity ~ +ve <i>R. rickettsii</i> = SFG +ve & +ve <i>R. typhi</i> = TG +ve	No information given on package insert	IgG: Sensitivity – 85% Specificity – 100% IgM: Sensitivity – 94% Specificity – 95%	IgG: Sensitivity – 100% Specificity – 100% IgM: Sensitivity – 97.4% Specificity – 86.8%
Limitations	Cross-reactivity within groups, Antibodies may not be present in first 2 weeks from onset of illness Convalescent testing required 1-2 weeks after Some patients never seroconvert from IgM to IgG IgG positive not enough to confirm a recent infection (antibodies can remain detectable for up to 12 months)	IgG & IgM: Cross-reactivity with other SFG <i>Rickettsia</i> thus unable to differentiate between causative species. IgM: Detects antibodies to protein antigens, will give negative results if the patient response is only to LPS antigen	Results + clinical evaluation + isolation = definitive diagnosis Lack of significant rise in antibody level does not exclude infection No detectable levels of IgG in very early samples ~ IgM recommended instead & following sample tested 14-21 days later IgG testing not recommended for neonate sera (<6 months) as IgG is passively transferred from mother to foetus For IgM testing, human IgG sorbant must be used to avoid false positives (rheumatoid factor) and false negatives (excess IgG antibodies)	Results in conjunction with clinical evaluation and other diagnostic procedures. Not intended to replace isolation. Lack of significant rise in antibody level does not exclude infection. No detectable levels of IgG in very early samples ~ IgM recommended instead & following sample tested 14-21 days later tested in parallel with original sample to determine seroconversion Requires paired sera IgG testing not recommended for neonate sera (<6 months) as IgG is passively transferred from mother to foetus

3.2.4 SAMPLE TESTING

All selected sera, acute and convalescent (where available) were tested using the available commercial kits without deviation from manufacturer's protocols, including screening cut-offs as set by manufacturer since regional baselines are not available.

3.2.4.1 Vircell IgG and IgM ELISA (*Rickettsia conorii*)

All kit reagents and frozen serum samples were removed from cold storage and allowed acclimate to room temperature (20 to 22°C) before use since use of kit components that were not at room temperature could compromise the assay results. All reagents, with the exception of the wash buffer solution, were supplied 'ready to use'. The 20x wash buffer was diluted 1:20 (v:v) with sterile distilled water (SDW) (50 mL of 20x wash buffer supplied with the kit in 950 ml SDW). The required number of wells were removed from the sealed foil bag and set out with four dedicated wells for controls required for the assay validation; i.e. one negative control (NC), one reactive control (RC) and two cut-off controls (CO¹ & CO²). Unused wells were returned to their foil bag containing a desiccant and resealed.

Samples and controls were pre-diluted in separate u-well ELISA plates (not included with the kit). For IgM tests, 25 µL of VIRCELL IgG sorbent (ref. S001) was added to the dilution plate for each of the test sera (excluding the controls), followed by the addition of 5 µL of sample and 75 µL of the serum diluent to each well. The dilutions were well homogenised and the entire 105 µL solution was added to the allocated wells. Controls for IgM tests were diluted as described for the IgG tests since the IgG sorbent was included in the control sera. For IgG tests 10 µL of sample/control was added to 200 µL sample diluent. Dilutions were well homogenised and 105 µL of the dilutions were transferred to allocated wells. The plate was covered and incubated at 37°C for 45 minutes, following which a wash cycle of 5 x 300 µL/well was run BioTek ELx50 microplate washer (BioTek

Instruments, Inc., USA). Excess liquid was tapped out of the wells and 100 µL of IgG conjugate solution was added to the wells, the plate was covered and incubated at 37°C for 30 minutes. The wash was repeated as before and 100 µL substrate solution was then added to the wells. The plate was incubated in the dark at room temperature for 20 minutes. Reactions were then stopped using 50 µL of stop solution (0.5 M sulphuric acid) and absorbances were read on a BioTek ELx800 microplate reader (BioTek Instruments, Inc., USA) with a 450 nm measuring filter and a 620 nm reference filter within 1 hour of the test completion. For the tests to have been deemed valid, optical densities (O.D.) of the controls (positive, negative and cut-off) had to fall within the required ranges shown in Table below.

Antibody indices were calculated by dividing the O.D. value obtained for the sample by the mean O.D. value for the cut-off controls (the duplicate well O.D. values were added together and divided by 2) and multiplied by 10.

$$\text{Antibody index} = \left(\frac{\text{sample O.D.}}{\text{cut off serum mean O.D.}} \right) \times 10$$

Interpretation of results is shown in the Table 3.3 below. Samples with indices below 9 (O.D. < 0.5) were considered negative, samples with indices above 11 (O.D. > 0.9) were considered positive and samples with indices between 9 and 11 were considered equivocal and were retested. All kit batch information was recorded onto a work card, attached to the microplate reader printout and filed. The results were transcribed onto an Excel spreadsheet, version 2016 (Microsoft, USA) spreadsheet for analysis.

Table 3.3. Vircell IgG/IgM *R. conorii* ELISA interpretation criteria.

Result	Index	Interpretation
Positive	> 11	IgG/IgM specific antibodies against <i>R. conorii</i> detected
Negative	< 9	No IgG/IgM (depending on procedure) specific antibodies against <i>R. conorii</i>
Equivocal	9 - 11	Retesting of the sample is required

3.2.4.2 Fuller Laboratories IgG SFG ELISA

All kit reagents and frozen serum samples were removed from cold storage and allowed to acclimate to room temperature before use as described previously. All reagents were supplied 'ready to use', except for the wash buffer which required that the PBS powder supplied be reconstituted in 1 L of SDW and that 2 mL of 25% Tween 20 (supplied with the kit) be added to the solution. This buffer was stored at 2 to 8°C for up to four months as per manufacturer recommendation. The relevant number of wells were removed from the sealed foil bag and set up as described previously with four dedicated wells for controls; one negative control, one positive control and two cut-off controls. The controls were diluted 1:10 (10 µL control diluted in 100 µL sample diluent) (v:v) since controls were packaged at a 1:10 dilution, and transferred to allocated wells (100 µL of the 1:100 final dilutions). Samples were diluted 1:100 (10 µL of sample added to 1000 µL sample diluent) (v:v), well homogenised and 100 µL of each dilution was transferred to allocated wells. The plate was covered and incubated at room temperature for one hour, following which a wash cycle of four repeats of 300 µL/well was run on a BioTek ELx50 microplate washer (BioTek Instruments, Inc., USA). Residual wash buffer was tapped out onto a piece of paper towel and 100 µL of conjugate solution was immediately added to the wells. The plate was again covered and incubated in the dark at room temperature for 30 minutes. The wash step was repeated and 100 µL substrate solution was added and incubated in the dark at room temperature for ten minutes. Finally, 100 µL of stop solution (0.5 M sulphuric acid) was added to halt reactions and the plate was read as described previously at 450 nm measuring filter and a 620 nm reference filter.

The cut-off calibrator provided, defines the intra-assay variation in the equivocal region to discriminate between reactive and non-reactive sera. Cut-off values measure the equivocal region of absorbance values between the highest and lowest values recorded for the controls. An index value is calculated by dividing the O.D. values of test sera by the Calibrator mean O.D. value. The calibrator is set at an index of 1.0 and indices from 0.8 to 1.2 are considered equivocal and samples within this range would require retesting. Indices > 1.2 are considered positive and those < 0.8 are considered negative (Table 3.4). All kit batch information was recorded onto a work card, attached to the microplate reader printout and filed. The results were transcribed onto an Excel spreadsheet, version 2016 (Microsoft, USA) spreadsheet for analysis.

$$\text{Antibody index} = \left(\frac{\text{Sample OD}}{\text{cutoff serum mean O.D.}} \right)$$

Table 3.4. Fuller Diagnostics IgG/IgM ELISA interpretation criteria.

Result	Index	Interpretation
Positive	> 1.2	IgG/IgM specific antibodies against <i>R. conorii</i> detected
Negative	< 0.8	No IgG/IgM (depending on procedure) specific antibodies against <i>R. conorii</i>
Equivocal	0.8 – 1.2	Retesting of the sample is required

This assay detects SFG-specific antibodies and is therefore unable to differentiate between SFG rickettsial species. Reactivity to typhus group or scrub typhus is in general, not detected. The prevalence of specific antibodies varies depending upon the geographic region and population being tested.

3.2.4.3 Focus Diagnostics IgG and IgM IFA (*Rickettsia rickettsii* and *R. typhi*)

The Focus Diagnostics (Langenhagen-Hannover, Germany) *Rickettsia* IgG and IgM indirect IFAs are intended for the detection and semi-quantitation of human

antibodies to SFG and TG *Rickettsia*. The assays utilize inactivated *R. rickettsii* antigens and *R. typhi* antigens and each slide contains eight wells, each well with two individual antigen spots (Figure 3.1).

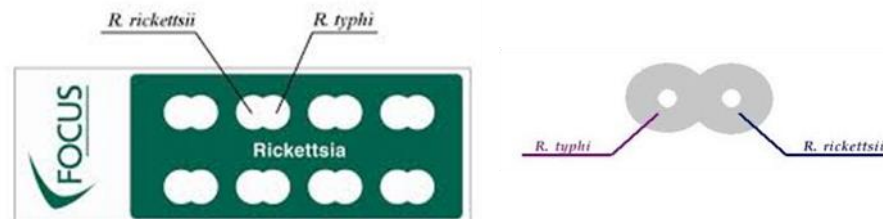


Figure 3.1. Focus Diagnostics IgG IFA slide depicting each well with two individual antigen spots; *R. rickettsii* on the left and *R. typhi* on the right.

IFA assays were set up in two (2) stages; firstly, patient sera were diluted in pre-treatment diluent and applied to the allocated wells, followed by the removal of the unbound serum antibodies and application of a fluorescein-labelled human IgM/IgG antibody (i.e. conjugate) to wells allowing for the visualization of the antigen-antibody complexes formed in the first stage.

Slides were removed from cold storage and allowed acclimate to room temperature prior to use. Positive and negative controls were included with each batch of samples run. Controls are supplied at screening dilution, thus 25 μ L of the relevant control (SFG or TG positive control and negative control) was applied directly to the allocated slide well. For each of the serum samples; a 1:4 (v:v) dilution of patient sera to reconstituted PBS was prepared. For the IgM assays, the 1:4 serum dilutions were further diluted 1:15 (v:v) in IgM pre-treatment diluent, a buffered isotonic solution of yolk sac suspension containing anti-human IgG; which removed both free and complexed IgG from the serum sample, to a final screening dilution of 1:64 (v:v). For the IgG assays 1:4 serum dilutions were further diluted 1:15 (v:v) in Yolk Sac Diluent to a final screening dilution of 1:64 (v:v). Final screening dilutions were allowed to incubate at room temperature for 15 minutes,

were briefly centrifuged, and 25 μ L of the diluted serum samples were applied to allocated wells overlaying the antigen spots. Slides were placed into a closed container with moist paper towel lining the bottom and incubated at 37°C for 30 minutes. The slides were then removed from the humid container and gently rinsed with a stream of PBS to remove any unbound serum antibodies, taking care to avoid sample mixing. Slides were then submersed in PBS for 10 minutes, briefly dipped in SDW, and placed on paper towel to allow for air-drying. A fluorescein-labelled antibody (25 μ L IgM or IgG (as applicable) conjugate) was then overlaid on the wells, the slides returned to the humid container, and re-incubated at 37°C for 30 minutes allowing antigen-antibody complexes to react with the fluorescein-labelled anti-human IgM/IgG. The wash step as previously described was repeated, and the slides were allowed to air dry in the dark. Several drops of mounting medium were applied to the slides and slides were covered with a 24x50 mm coverslip. Air bubbles and excess mounting medium were removed with absorbent paper. Wells were viewed at a final magnification of 200X on the Olympus BX41 fluorescence microscope (Olympus Corporation, Tokyo, Japan). Controls were viewed first and sample fluorescence was then compared to that of the controls. To ensure that optimum fluorescence was observed, the slides were kept in the dark and read on the same day. Slides were blindly read by a second operator to ensure objective reading was achieved. Results were independently recorded onto work cards (Figure 3.2) and the two operators' results were compared and discrepancies were resolved by a third person reading the discrepant results. Positive reactions appeared bright apple-green cytoplasmic fluorescence against a background of orange to red yolk sac matrix. Working cards were filed and results were transcribed into an Excel spreadsheet, version 2016 (Microsoft, USA) for analysis.

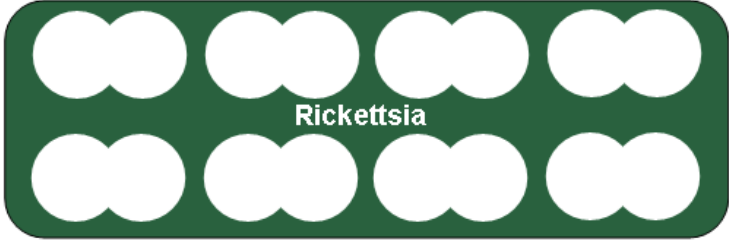
Date:	
Lot:	
Exp:	
By:	

Figure 3.2. Work cards prepared for Focus Diagnostics IgG and IgM IFAs. Printed in duplicate to be completed by both operators independently. Results compared and discrepancies resolved.

3.2.5 DATA ANALYSIS AND STATISTICAL INFERENCE

As described in section 3.2.1., 48% (63/132) of the selected samples were negative (33 AFI samples and 30 KNP samples), and 52% (69/132) were positive (12 AFI samples and 57 KNP samples) based on the Vircell IgG IFA. The null hypothesis suggests that there would be no significant difference in the results obtained between the Vircell *R. conorii* IgG IFA and any of the other three IgG assays tested in this study.

Panel results were captured on an Excel 2016 (Microsoft) spreadsheet and basic descriptive data analysis was carried out on Excel (i.e. percentage agreement between the assays and frequency tables). The freeware JASP Version 0.9.2 (JASP Team, 2018) computer software was used to carry out Chi-Squared tests and determine p-values. P-values were used to determine whether the difference in results between any two kits compared was statistically significant at 95% confidence (i.e. any p-value > 0.05 indicates that the difference is not significant).

3.3 RESULTS

3.3.1 IgG ASSAY COMPARISON

3.3.1.1 IFA comparison: Vircell *R. conorii* IFA vs Focus Diagnostics *R. rickettsia* IFA

Results obtained using the Vircell IFA were compared to those obtained using the Focus Diagnostics IFA. It was shown that there was a 68% (90/132) agreement between the two (2) assays. In 15% (20/132) of the tests called positive on the Focus Diagnostics IFA, were found to be negative on the Vircell IFA, and in 17% (22/132) of the samples testing positive on the Vircell IFA were found to be negative on the Focus Diagnostics IFA kit. The Chi-Squared test value was 17.43, with one (1) degree for freedom and p-value < 0.001 indicating a significant difference between the two (2) IFA assay results. (Table 3.5).

Table 3.5. Contingency table and Chi-Squared test comparing the reference method Vircell *R. conorii* IgG IFA to the Focus Diagnostics *R. rickettsii* IgG IFA

	REFERENCE METHOD: Vircell IgG IFA		
Focus SFG IgG IFA	NEG	POS	Total
NEG	43	22	65
POS	20	47	67
Total	63	69	132

Chi-Squared Tests			
	Value	df	p-value
χ^2	17.43	1	< 0.001
N	132		

*df: degrees of freedom

3.3.1.2 Vircell *R. conorii* IFA vs ELISA

The Vircell IFA was compared with the Vircell ELISA and it was shown that only 66% (87/132) of the samples yielded the same results for both the testing platforms, despite the manufacturer and target antigen (i.e. *R. conorii*) being the same for the two (2) assays. The ELISA yielded four (4) equivocal results (i.e. indices between 9 and 11, optical densities 0.9 – 1.1), two (2) of which were positive and two (2) negative according to the Vircell IFA. The Vircell ELISA provided negative readings for 9% (12/132) of the samples that were considered

positive by IFA, and ~24% (29/132) positive by ELISA that were reportedly negative by IFA. The Chi-Squared statistic was 16,9 with two (2) degrees of freedom, p-value of < 0.001 indicating a significant difference between the two (2) tests (Table 3.6).

Table 3.6 Contingency table and Chi-Squared test comparing the reference method Vircell *R. conorii* IgG IFA to the Vircell *R. conorii* IgG ELISA.

Vircell IgG EIA	REFERENCE METHOD: Vircell IgG IFA		
	NEG	POS	Total
NEG	32	12	44
POS	29	55	84
EQV	2	2	4
Total	63	69	132

Chi-Squared Tests			
	Value	df	p-value
X ²	16.90	2	< 0.001
N	132		

*df: degrees of freedom

3.3.1.3 Vircell *R. conorii* IFA vs. Fuller Laboratories SFG ELISA

The Vircell *R. conorii* IFA was then compared with the Fuller Laboratories *R. rickettsii* ELISA. In this comparison it was shown that only 63% (84/132) of the sample results were consistent between the two (2) assays. The Fuller Laboratories ELISA provided 38 (29%) positives that were reportedly IFA negative as well as an additional three (3) equivocal results that were considered negative by IFA. Based on this comparison it would appear that either the ELISA cut-offs are set too low, thus providing false positives or positives were being missed by the IFA technique. The Chi-Squared statistic was 16.28 with two (2) degrees of freedom and a p-value of < 0.001 indicated a significant difference between the two (2) assays (Table 3.7).

Table 3.7 Contingency table and Chi-Squared test comparing the reference method Vircell R. conorii IgG IFA to the Fuller Laboratories R. rickettsii IgG ELISA.

	REFERENCE METHOD: Vircell IFA IgG		
Fuller IgG EIA	NEG	POS	Total
NEG	22	7	29
POS	38	62	100
EQV	3	0	3
Total	63	69	132

Chi-Squared Tests			
	Value	df	p-value
χ^2	16.28	2	< 0.001
N	132		

*df: degrees of freedom

3.3.1.4 Vircell R. conorii ELISA vs. Fuller Laboratories SFG ELISA

To determine the rate of agreement between the two (2) IgG ELISAs, the results were compared in a 3x3 Chi-Squared test. The two (2) ELISAs provided the same result in 110 of the 132 (83% agreement) samples tested. The Fuller ELISA indicated that 100/132 of the samples were IgG positive whereas the Vircell indicated 84/132 of the samples were positive. For 82 of the positives given by either of the two (2) ELISAs there was agreement between the assays. The Chi-Squared statistic for the two (2) ELISAs is 70.50 with four (4) degrees of freedom and a p-value of < 0.001 indicating a significant difference between the two (2) assays (Table 3.8).

Table 3.8. Contingency table and Chi-Squared test comparing the Vircell R. conorii IgG ELISA with the Fuller Laboratories R. rickettsii IgG ELISA.

	Fuller IgG EIA			
Vircell IgG EIA	NEG	POS	EQV	Total
NEG	28	14	2	44
POS	1	82	1	84
EQV	0	4	0	4
Total	29	100	3	132

Chi-Squared Tests			
	Value	df	p-value
χ^2	70.50	4	< 0.001
N	132		

*df: degrees of freedom

3.3.2 IgM ASSAY COMPARISON

IgM antibodies are typically the first to appear following an exposure to a given antigen, and are generally associated with an acute or recent infection and not expected in clinically 'well' individuals. For this reason, the kits could not be compared with the Vircell IgM IFA as IgM results, as this assay was not considered necessary nor applicable to the KNP clinically 'well' samples in their primary analyses. The IgM assays were also only selectively carried out for some of the AFI study samples, therefore there were incomplete records available for the Vircell IgM IFA. It is for this reason that kits could not be compared with those of the initial assays conducted on the samples collected for the previous studies. The samples were; however still tested by the three (3) assays to determine whether different results would be obtained between the three (3) assays. As a null hypothesis, it was expected that kits would provide consistent results with no significant difference between them. The same panel of samples described for the IgG assay comparisons above was used for the IgM tests, with the exception of one (1) AFI sample omitted due to insufficient sample volume for all required testing (n=131).

3.3.2.1 *Focus Diagnostics R. rickettsii IgM IFA vs Fuller Laboratories R. rickettsii IgM ELISA*

When the Focus IgM IFA results were compared to the Fuller Laboratories IgM ELISA, the rate of agreement of the results was 91% (118/131). The Fuller Laboratories ELISA provided only two (2) positive results and the Focus Diagnostics IFA had only one (1) positive result - these positives did not coincide as they were for three (3) different samples; i.e. the positive results were not common to both assays. The Fuller EIA provided ten (10) equivocal results indicating that the O.D. value indices for those samples fell within the 0.8 to 1.2 range even after being repeated, and could therefore not be definitively called either positive or negative. In this comparison, the Chi-Squared statistic was 0.102

with two (2) degrees of freedom and a p-value of 0.950 indicating that there was no significant difference between the two (2) assays (Table 3.9).

Table 3.9. Contingency table and Chi-Squared test comparing the Focus Diagnostics SFG IgM IFA with the Fuller Laboratories SFG IgM ELISA.

Fuller EIA IgM	Focus IFA IgM SFG		
	NEG	POS	Total
NEG	118	1	119
POS	2	0	2
EQV	10	0	10
Total	130	1	131

Chi-Squared Tests			
	Value	df	p-value
χ^2	0.102	2	0.950
N	131		

*df: degrees of freedom

3.3.2.2 Focus Diagnostics *R. rickettsii* IgM IFA vs Vircell IgM EIA

The Focus Diagnostics IgM IFA had a ~ 95% (124/131) rate of agreement with the Vircell IgM EIA. Both kits called 124 of the panel samples negative, whilst the positives showed had no agreement between the two (2) assays. The Vircell EIA provided (4) positive results and two (2) equivocal results that were negative by IFA, and the Focus IFA had one (1) positive that tested negative by EIA. The Chi-Squared statistic was 0.048 with two (2) degrees of freedom and a p-value of 0.976 indicating that there was no significant difference between the two (2) assays (Table 3.10).

Table 3.10. Contingency table and Chi-Squared test comparing the Focus Diagnostics SFG IgM IFA with the Vircell *R. conorii* IgM ELISA.

Vircell IgM EIA	Focus IFA IgM SFG		
	NEG	POS	Total
NEG	124	1	125
POS	4	0	4
EQV	2	0	2
Total	130	1	131

Chi-Squared Tests			
	Value	df	p-value
χ^2	0.048	2	0.976
N	131		

*df: degrees of freedom

3.3.2.3 Fuller Laboratories IgM EIA vs Vircell IgM EIA

There was an 88% (115/131) agreement between the two (2) EIA assays. Both assays provided 114 negative results for the panel and one (1) equivocal result common to both assays. Positive and equivocal (except for 1) results did not correlate between the two (2) assays. The Chi-Squared statistic was 5.542 with four (4) degrees of freedom and a p-value of 0.236, indicating that the two (2) assays were not significantly different despite the low agreement in positive results (Table 3.11).

Table 3.11. Contingency table and Chi-Squared tests comparing the Fuller Laboratories IgM ELISA with the Vircell *R. conorii* IgM ELISA.

Vircell IgM EIA	Fuller EIA IgM			
	NEG	POS	EQV	Total
NEG	114	2	9	125
POS	4	0	0	4
EQV	1	0	1	2
Total	119	2	10	131

Chi-Squared Tests			
	Value	df	p-value
X ²	5.541	4	0.236
N	131		

*df: degrees of freedom

3.4 DISCUSSION

In summation, the null hypothesis was rejected for all the IgG kit comparisons demonstrated by all statistical analyses indicating significant differences between the Vircell *R. conorii* IFA and each of the other assays evaluated. It was particularly surprising that the two tests produced by the same manufacturer (i.e. Vircell *R. conorii* IFA vs Vircell *R. conorii* ELISA) also produced significantly different results, with the objective ELISA demonstrating a higher number of positive results than the subjective IFA. It was also found to be surprising that when parallel technologies were compared to each other (i.e. Vircell *R. conorii* IFA vs Focus Diagnostics *R. rickettsia* IFA, and Vircell *R. conorii* ELISA vs. Fuller Laboratories SFG ELISA), results were again found to be significantly different. This significant difference was however, not observed between the IgM assay comparisons.

Serology remains one of the most heavily relied upon means of diagnosing Rickettsial diseases, however result interpretation is reliant on clinically consistent presentation of illness and appropriate epidemiological data for the region (Stewart and Stewart, 2021). To further complicate diagnosis, the phase of rickettsial disease is important for testing outcomes as serological evidence of rickettsial infection may not be revealed until the second week of disease (Stewart and Stewart, 2021). At the time of this study, few published evaluations of Rickettsial serological diagnostic assays were available (Kantso et al., 2009, Jensenius et al., 2004b, Broadhurst et al., 1998). Immunofluorescence assays were still considered the reference method for diagnosis of *Rickettsia* infections, despite variability in sensitivity and specificity (Jensenius et al., 2004b, Portillo et al., 2017). According to various authors, IFA sensitivity range from 42% to 100% (Stephen et al., 2018). There are several commercially-manufactured assays used by laboratories worldwide, with little data available for comparisons between similar assays produced by various kit manufacturers to determine agreement of results between these kits. A 2009 study conducted in Denmark compared two (2) commercially-available IFA kits to the WF and each other, and found 100 % agreement between the kits when they dichotomized results in accordance with manufacturer-recommended cut-off levels (Kantsø et al., 2009). As expected, the Denmark study also reported having adjusted the cut-off criteria to what was deemed appropriate for the Danish population based on the titres of presumably healthy blood donors (Kantsø et al., 2009). In contrast, our study indicated a significant difference between two (2) IgG IFA kits developed and produced by different manufacturers. Had it been possible for us to adjust our cut-off values to our local population, the picture may have been different, although this cannot be assumed. A confounding factor of serological diagnoses of rickettsioses is the occurrence of pre-existing levels of antibodies in an 'at-risk' populations that are reactive to a particular pathogen other than the actual disease under investigation (Paddock, 2013). It is therefore highlighted that setting study population-specific cut-offs for a particular area is perhaps valuable. This task is however, not as simple to

perform. Review of the literature indicates that in the majority of serological studies, the cut-off titres used had no clear justification for the values used (Paddock, 2013). The majority of the authors determined that no single antibody titre can dependably be considered diagnostic, unless prior studies have been achieved to determine seroprevalence in the healthy population of a particular area (Paddock, 2013).

It is important to note that serological discrepancies even occur when the same samples are tested by different laboratories (Paddock, 2013). This was also demonstrated in a related inter-laboratory comparison study conducted in South African laboratories (Trataris-Rebisz et al., 2019). The inter-laboratory comparison involved three (3) major South African laboratories that routinely offer Rickettsial serological diagnostics and it was shown that there was a lack of consistent agreement between the laboratories. In a panel of ten (10) samples, selected for being consistently either positive or negative over four (4) testing platforms, the overall agreement between the laboratories was only 83% and 77% for IgG and IgM respectively, despite all being tested by commercially-available IFAs (Trataris-Rebisz et al., 2019). The panel for inter-laboratory study was compiled from the samples of this study that were in complete consensus over all four (4) of the assays used. The fact that in the hands of other operators, in different laboratory settings, different results were produced for some of the samples, further highlights the difficulty of *Rickettsia* serological diagnostics in South Africa. It is concerning that, in a moderate study sample size of 132, the two (2) IFA assays only agreed 68% (90/132) of the time. This was not however, isolated to the IFA assays, since the two (2) IgG ELISAs were also found to produce significantly different results when they were compared to each other and to the primary test results obtained in the initial studies from which the samples were selected, albeit a higher rate of agreement (83% - 110/132) than the rate between the two (2) IFAs. It has been reported that ELISAs perform better than IFAs, provided geographical cut-off titres for different regions are known and used for analysis (Stephen et al., 2018).

A major struggle lies in determining which assay will provide the most accurate representation of the true situation, irrespective of where the patient resides. The lack of standardization, variable cut-off titres for endemic regions, the necessity for paired sera to be tested concurrently, the high cost of testing, and subjective endpoints for IFAs are causes for concern without a clear resolution currently available (Paddock, 2013). Published literature has widely criticized the reference method for its various limitations (Luce-Fedrow et al., 2015). One (1) such limitation is that ATBF cases are often being missed by SFG IFAs (Jensenius et al., 2004b) and there are no specific serum immunofluorescence test for *R. africae* commercially available (Johnson et al., 2019). The commercially purchased kits that were used in this study rely on SFG antibody cross-reactivity to establish a diagnosis, and although serological cross-reactivity is common among members of the SFG rickettsiae including *R. africae* (Fournier et al., 2002, Jensenius et al., 2003, Johnson et al., 2019) antibody titre variability associated with *Rickettsia* infections complicate serological diagnostics. This has been shown in *R. africae* cases where a rise in antibody titres is usually delayed (Jensenius et al., 2004a, Luce-Fedrow et al., 2015). Although this factor was not relevant in for this study, since residual samples from previous studies were used, it becomes increasingly more relevant when determining a diagnostic strategy for a region, which is essentially a goal of this comparative study. Furthermore, IFA cross-reactivity is a double-edged sword, since the SFG rickettsial diagnostics is reliant on cross-reactivity to detect the various SFG rickettsias, but IFAs have also shown cross-reactivity with TG rickettsiae as well as *Coxiella burnetii* and *Bartonella* species (Luce-Fedrow et al., 2015).

Additional challenges relating to IFAs include technical limitations. IFAs require experienced technicians to read the results and are highly subjective to the operator reading the slides (Fournier et al., 2002, Luce-Fedrow et al., 2015). Some operators tend to call slides negative unless the observed fluorescence is vibrant,

missing true positives, whereas other operators may call even the slightest observed fluorescence positive, overcalling negatives as false positives. This can be attributed to fading of the fluorescence through prolonged exposure to light, or background fluorescence respectively. The two (2) IFAs used provided consistently concordant results for 47 of the 69 positive samples, however, 22 of the 69 Vircell positives were discrepant. The significantly different results obtained obviously complicate rickettsia diagnostics, as laboratories looking to employ one (1) of these commercial assays in their setting would face the conundrum of which kit to select. In order to compensate for IFA limitations, various authors have made recommendations; for example, systematization of IFA methodologies, standardization of cut-off titres, reporting of types/brands of antigens/antibodies used, establishing background serology levels in the healthy populations in endemic countries and examination of host and vector species to characterize new rickettsial strains in a population (Luce-Fedrow et al., 2015).

The two (2) IgG ELISAs were also found to be significantly different to each other and to the Vircell *R. conorii* IFA. The ELISAs showed agreement in 82 SFG positive results, 13 more than those of the Vircell IFA's (i.e. reference test) 69 positive results. This indicates that either the IFA is failing to diagnose 13/82 IgG positive cases, or the ELISA IgG cut-off values are too low for the sampled population. Enzyme-linked immunosorbent assays are reportedly attractive for their ability to screen a large number of serum samples at a time (up to 91 samples per plate), they are relatively inexpensive, technically easy to perform, results are reproducible and objectively performed and read (i.e. interpretation is not dependent on operator perception but is based on measurable optical density) (Luce-Fedrow et al., 2015, Fomda et al., 2021). The ELISA method has also been shown to be more effective at identifying lower level antibodies in comparison with IFAs (La Scola and Raoult, 1997, Luce-Fedrow et al., 2015). A limitation of ELISAs, shared with other serological assays, is the inability to discriminate between rickettsial and oriental species (Luce-Fedrow et al., 2015).

This study had several limitations associated with the use of the residual samples, including; the repeated freeze-thawing of specimens contributing to the degradation of antibodies which may have been present in the samples, low sample volumes available for testing meant duplicate or repeat testing could not be performed and original tests conducted using the Vircell IFA were carried out in a different laboratory, by a different operator so it is unclear whether the results would have been read the same way had the initial tests been read and conducted by the same operator as with the rest of the assays compared. Another limitation of this study is that samples were tested at manufacturer screening dilutions since geographically relevant epidemiological data is not readily available. However, since the objective was to determine whether the commercially available assays provide comparable results, specific titres were not considered paramount to this study.

The residual samples used for this study, were remnant of an afebrile illness study and an occupational risk factor study, therefore it was not known whether the samples were indicative of a current or recent rickettsial exposure (i.e. circulating bacteraemia) or previous exposure and infection. The former was assumed for the afebrile illness samples and the latter for the occupational risk factor study samples. Due to the lack of characterized, i.e. known positive and negative, South African context material, it was not possible to test positive and negative predictive values. It therefore remains unknown whether the IFA is underrepresenting the positives (i.e. false negatives), or the ELISAs are over-representing the positives (i.e. false positives).

The use of residual quality assurance samples was considered for validation purposes, however, this would not necessarily be a true indication of kit performance in the South African context since the available quality assurance programs are prepared internationally where different *Rickettsia* species are endemic. This remains a limitation for all South African laboratories offering

Rickettsia diagnostics and requiring characterised samples for validation purposes.

3.5 CONCLUSION

The significantly different results obtained between the various assays are indicative of the complexity of *Rickettsia* serological diagnosis. It is therefore prudent to triangulate assay readings with relevant clinical history, travel/exposure history, presence of an eschar, and/or PCR positive result (if available). It is also important to note that for current or recent infections to be confirmed, both acute and convalescent samples must be tested in tandem, whereby a marked rise in antibody titres is observed between acute and convalescent samples. Furthermore, it should be noted that *R. africae* is highly prevalent in South Africa, and may be the causative agent of the infection, but since *R. africae* antibodies may only be detectable 25 to 28 days after infection, it is recommended that a third sample be collected and tested for *R. africae* as the causative agent (Luce-Fedrow et al., 2015). It is vital for laboratories to validate diagnostic assays using population-derived clinical material in order to determine suitable population-based cut-off criteria. Clinicians should not delay placing a patient on treatment if there is a negative serological result from a patient sample taken early in the infection presentation as *Rickettsia* antibody responses are generally seen 5 to 10 days after onset of illness. It is recommended that treatment be ordered and a convalescent sample be taken and tested later to observe a four-fold rise in antibody titres (McQuiston et al., 2014).

CHAPTER 4 - SPOTTED FEVER RICKETTSIOSIS IN SOUTH AFRICA: EVALUATION OF LABORATORY DIAGNOSTIC CAPACITY AND INTER- LABORATORY COMPARISON FOR SEROLOGICAL TESTING

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4.1 INTRODUCTION

The rickettsiae belong to a group of Gram-negative, arthropod-borne, obligate intracellular bacteria that cause a wide spectrum of diseases, varying from mild and self-resolving to fatal infections (Lepidi et al., 2006, Blanton and Walker, 2017, Parola et al., 2005a, Merhej et al., 2014). With the enhancement of diagnostic methodologies, and specifically the use of molecular techniques, and improved ecological surveillance, the importance of rickettsiae as neglected emerging infectious diseases globally has come to the fore (Blanton and Walker, 2017, Essbauer et al., 2018, Parola et al., 2005a, Merhej et al., 2014, Bizzini et al., 2015). The rickettsiae are distributed globally and disease occurrence is associated with the distribution of arthropod vectors (Merhej et al., 2014). The latter includes various species of ticks, lice, fleas, mites and mosquitoes (Merhej et al., 2014). Rickettsioses do not have any specific pathognomonic signs, but rather a range of generalized signs and symptoms which may include fever, rash, lymphadenopathy and headaches (Faccini-Martinez et al., 2014, Stewart and Stewart, 2021). These symptoms together with a history of insect bite (mostly ticks), one (1) to two (2) weeks before the development of illness, in the presence or absence of a notable eschar (or tache noire), are considered highly suggestive of the diagnosis of rickettsiosis (Schjørring et al., 2020).

Tick bite fever commonly occurs in southern Africa (Pretorius and Birtles, 2004b, Ndip et al., 2004, Raoult et al., 2001, Ndip et al., 2011, Frean et al., 2008). Seroprevalence studies have indicated up to 80% positivity in specific communities, whilst the disease is often reported in travellers to southern Africa (Socolovschi et al., 2012b, Jensenius et al., 2003a, Simpson et al., 2018). There are mainly two *Rickettsia* species associated with TBF in South Africa, namely *R. africae* and *R. conorii*, causing ATBF and MSF respectively (Frean et al., 2008, Jensenius et al., 2003a, Jensenius et al., 2003c, Pretorius and Birtles, 2002). *Rickettsia africae* is reportedly transmitted by *Amblyomma* (or 'bont') tick species, *A. variegatum* and *A. hebraeum*, whereas *R. conorii* has been associated with the brown dog tick, *Rhipicephalus sanguineus* (Pretorius and Birtles, 2004b, Jensenius et al., 2003a, Frean et al., 2008). Single cases of TBF in South Africa associated with *R. aeschlimannii* (transmitted by *Rhipicephalus appendiculatus*) (Pretorius and Birtles, 2002) and *R. mongolotimonae* have also been reported (Pretorius and Birtles, 2004b, Frean and Grayson, 2019). The clinical presentation of TBF associated with these different rickettsiae are however very similar and identification of aetiological agent is not required for patient management (Pretorius and Birtles, 2004b, Jensenius et al., 2003a, Frean et al., 2008).

The clinical syndrome of TBF is mostly uneventful and resolves with antibiotics, but fatal cases have also been reported in patients with and without co-morbid features (Lepidi et al., 2006, Jensenius et al., 2003a, Frean and Grayson, 2019). Fatalities are often associated with delayed clinical recognition and antibiotic treatment. Although the clinical suspicion and patient response to doxycycline or tetracycline treatment are often used to diagnose cases of TBF, specific laboratory testing for TBF can be an important tool to direct patient management, especially in severe cases (Jensenius et al., 2003a, Frean et al., 2008, Pretorius and Birtles, 2002). The differential diagnoses of TBF is vast, including CCHF virus, meningococemia, rubella, measles, secondary syphilis, disseminated gonococcal

disease, enterovirus or arbovirus infections, anthrax, brucellosis, cat scratch disease, leptospirosis, typhoid, immune complex vasculitis or drug reactions, and as such, the value of laboratory investigation should not be underestimated (Frean et al., 2008).

There are several factors to consider for the laboratory diagnosis of TBF. Conventional testing including the WF test (La Scola and Raoult, 1997, Fomda et al., 2021) is no longer recommended as it lacks in sensitivity and specificity, but is still practiced in some routine laboratories particularly in resource constrained settings (Frean et al., 2008). The IFA, as previously mentioned, is considered the gold standard for TBF diagnosis and is the test most commonly performed in routine diagnostic laboratories (La Scola and Raoult, 1997). This form of testing is rapid and sensitive, but antibody cross-reactivity between rickettsial species renders the test unsuitable for determining the exact species causing the infection. Other proteo-bacterial antigens may also cross-react with rickettsial antigens and this should be taken in consideration for interpretation of results. Importantly, anti-rickettsial antibodies only develop after the first week of illness, and may therefore be uninformative during acute and early stages of disease. Results need to be interpreted with context of patient clinical history since negative findings may inadvertently misdirect the diagnosis (La Scola and Raoult, 1997). Indirect immunofluorescence, based on microscopic observation, requires highly skilled laboratorians to interpret the reading of the assay. A highly sensitive and reproducible ELISA for anti-*rickettsia* antibody detection is also commercially available, and allows for the differentiation of IgG and IgM antibodies (La Scola and Raoult, 1997). Enzyme-linked immunosorbent assays have been shown to be more sensitive than IFAs in detecting low-level antibodies, and are suitable for demonstrating rising antibody titres (La Scola and Raoult, 1997, Fomda et al., 2021). Increasingly, molecular techniques are being used to detect rickettsial DNA in eschar swabs, skin biopsies or blood (La Scola and Raoult, 1997, Portillo et al., 2017). These tests are, however, not as commonly available as serological testing. Culturing of rickettsiae is also not routinely available, as it requires the use of BSL-

3 facilities and *in vivo* techniques (Bizzini et al., 2015). Other commercially available diagnostic tests include other agglutination assays, indirect hemagglutination, immunoperoxidase assay, CF test, WB, and line probe assays (La Scola and Raoult, 1997). These assays vary in diagnostic sensitivity and specificity.

In general, there is a lack of standardisation across the different testing platforms and between laboratories offering these tests (La Scola and Raoult, 1997). This study reports on the status quo of laboratory testing for TBF in South Africa and reports on the findings of an ILC for serological tests.

4.2 MATERIALS AND METHODS

4.2.1 STUDY PARTICIPANTS

Five (5) pathology service providers (hereafter laboratories) known to offer routine TBF testing were invited to participate in this study. Laboratories included serviced both the private and state healthcare sectors in South Africa.

4.2.2 ETHICS STATEMENT

Ethical clearance for the use of the archival material was obtained prior to the study (University of Witwatersrand Human Research Ethics Committee, M120667 (amended) and the University of Pretoria Human Research Ethics Committee, 222/2017) (please see supplementary materials – Appendices 3.2 and 3.3).

4.2.3 QUESTIONNAIRE

Participating healthcare laboratories were provided with an electronic questionnaire requesting information regarding the availability of TBF diagnostic testing. Participation was voluntary and a period of two (2) weeks was allowed for

respondents to submit their answers. It was requested that senior, qualified staff complete the questionnaire. The self-administered questionnaire comprised of 53 questions divided into five (5) sections, requesting information on serological and molecular tests offered as well as technical details relating to the tests (see Appendix 4.1). Information regarding the quality assurance program was also requested. The data were subsequently captured and analysed in an Excel spreadsheet, version 2016 (Microsoft, USA).

4.2.4 INTER-LABORATORY COMPARISON

The ILC was conducted to gauge the performance and agreement of serological results for TBF for a standardised clinical specimen panel. Laboratories were invited to participate and indicated their willingness to participate in the ILC, in the questionnaire mentioned above. Three (3) of the five (5) laboratories offering TBF testing agreed to be included. A single ILC panel was supplied to each laboratory and testing was performed through their routine protocols. Participating laboratories were requested to test the panel per routine protocol for TBF serological testing (for both anti-rickettsia IgG and IgM) and to report the results to the provider within four (4) weeks of receipt (see Appendix 4.2).

4.2.4.1 *Panel*

The panel was numerically coded and had no association with the original treatment system. The samples derived from clinical material collected through previous research studies or clinical submissions to the CEZPD, NICD (Ethics clearance certificate for this study: M120667 (amended – WITS HREC) & 222/2017 (UP HREC)). The serum panel was characterised through repeat testing with four (4) different commercially available assays for rickettsia IgG and IgM determination: Vircell IgG and IgM *R. conorii* IFA (Vircell, Spain), Vircell IgG and IgM *R. conorii* ELISA, Focus Diagnostics IgG and IgM SFG and TG IFA (Focus Diagnostics, USA), and Fuller Laboratories IgG and IgM SFG ELISA (Fuller

Laboratories, USA) (see chapter 3). Four (4) of the ten (10) samples provided tested *Rickettsia* IgG positive, and the remaining six (6) tested IgG negative. All ten (10) samples tested negative for IgM across all assays used (Table 4.1 & Table 4.2).

Only specimens that gave consistent, non-equivocal results were selected. Selected sera were homogenised by brief vortex mixing and centrifuged, followed by aliquoting and storage at -20°C . A subset of aliquots was subjected to stability testing, following storage at 4°C and -20°C , at two (2)-week intervals (i.e. distribution date, mid-point and closing of the ILC). The panel was numerically coded with no identifiers linking a sample to patient information or the original study for which the samples were collected. The ILC panels were handled and transported on ice and in accordance with international regulations for the transport of diagnostic samples. Accompanying documentation included handling (including safety), storage and testing instructions, as well as a standardised response form (see Appendix 4.2). The expected IgG result for samples 1, 3, 5, 6, 8, and 10 is negative and positive for samples 2, 4, 7, and 9. All samples were expected as IgM negative due to non-reactivity observed across four (4) test platforms.

Table 4.1 Panel sample IgG results obtained at CEZPD using Vircell IFA and ELISA, Focus SFG IFA and Fuller ELISA.

Sample Number	Expected IgG result/s	Vircell IgG IFA	Vircell IgG ELISA	Focus SFG IgG IFA	Fuller IgG ELISA
2018/01 – 001	NEG	NEG	NEG	NEG	NEG
2018/01 – 002	POS	POS	POS	POS	POS
2018/01 – 003	NEG	NEG	NEG	NEG	NEG
2018/01 – 004	POS	POS	POS	POS	POS
2018/01 – 005	NEG	NEG	NEG	NEG	NEG
2018/01 – 006	NEG	NEG	NEG	NEG	NEG
2018/01 – 007	POS	POS	POS	POS	POS
2018/01 – 008	NEG	NEG	NEG	NEG	NEG
2018/01 – 009	POS	POS	POS	POS	POS
2018/01 – 010	NEG	NEG	NEG	NEG	NEG

Table 4.2 Panel sample IgM results obtained at CEZPD using Vircell IFA and ELISA, Focus SFG IFA and Fuller ELISA.

Sample number	Expected IgM result/s	Vircell IgM IFA	Vircell IgM ELISA	Focus SFG IgM IFA	Fuller IgM ELISA
2018/01 – 001	NEG	NEG	NEG	NEG	NEG
2018/01 – 002	NEG	NEG	NEG	NEG	NEG
2018/01 – 003	NEG	NEG	NEG	NEG	NEG
2018/01 – 004	NEG	NEG	NEG	NEG	NEG
2018/01 – 005	NEG	NEG	NEG	NEG	NEG
2018/01 – 006	NEG	NEG	NEG	NEG	NEG
2018/01 – 007	NEG	NEG	NEG	NEG	NEG
2018/01 – 008	NEG	NEG	NEG	NEG	NEG
2018/01 – 009	NEG	NEG	NEG	NEG	NEG
2018/01 – 010	NEG	NEG	NEG	NEG	NEG

4.2.5 DATA ANALYSIS

Results reported were summarised in an Excel spreadsheet 2016. The agreement of results submitted by the participants with the expected result was calculated as a proportion of results consistent with the expected results. Likewise, the overall agreement of results for IgG and IgM, respectively, was calculated as the proportion of all results in agreement with the expected results.

Results were analysed through calculating congruence of the received and expected results. Analysis of IgG and IgM results were calculated separately and percentages were calculated as number of laboratories reporting results congruent with the expected ones divided by the number of participating laboratories (n=3). Therefore, if 2/3 labs had matching results with the expected, the percentage would be calculated as 67%, if only one (1) laboratory reported congruent results, the percentage was reported as 33%. The overall IgG and IgM congruencies for the ILC were calculated based on the averages calculated over the ten (10) samples.

$$\text{Overall congruency \%} = \left(\frac{\# \text{ congruent labs}}{\# \text{ participating labs}} \right) \times 100$$

4.3 RESULTS

4.3.1 QUESTIONNAIRE

All participating laboratories were compliant with the two (2)-week questionnaire and four (4)-week ILC result submission deadlines. Responses to the questionnaire were received from all participating laboratories (n=5), including both the private and state sectors of the South African health care system. Serological testing for anti-rickettsia IgG and IgM was offered by all the laboratories, with three (3) of the laboratories indicating that they also offered PCR tests. All laboratories offering *Rickettsia* serology used commercially-available IFA kits for IgG and IgM antibody detection. Four (4) of the five (5) laboratories used the Focus Diagnostics IFA kit and one (1) used the Vircell IFA kit. All the serological tests were based on *R. conorii* antigens, although *R. africae* would also be detected due to antigenic cross-reactivity. All respondents indicated that the commercial kits were subject to in-house validation for diagnostic use. Four (4) of the five (5) laboratories indicated that the *Rickettsia* serological testing offered was accredited to international

standards through the South African National Accreditation System (SANAS). Three (3) of the four (4) accredited laboratories conducted routine proficiency testing through the Royal College of Pathologists of Australasia.

In addition to serology, three (3) laboratories indicated that they also offered PCR to detect rickettsial DNA targets. Importantly, PCR testing was not available for state facilities. Of the three (3) labs that offered PCR diagnostic assays, one (1) used an in-house-developed method that employs previously-published primers, and the other two (2) laboratories used a commercially-available real-time PCR that targets the citrate synthase (*gltA*) gene of all *Rickettsia* species. The laboratory that employs in-house methods has two (2) real-time PCRs available; one (1) that targets the *gltA* gene for SFG rickettsias and another that targets the *gltA* gene for TG group rickettsias. Multiple sample types (including blood, and eschar biopsies and swabs) may be tested using the commercial kit, but only plasma or serum was recommended for the in-house methods. Two (2) of the three (3) laboratories that offer rickettsial PCR used in-house-validated methods. None of the laboratories employed external proficiency testing or used accredited PCR methods.

4.3.2 INTER-LABORATORY COMPARISON

Three (3) laboratories participated in the serology ILC. Laboratory 1 used the Vircell IgG and IgM *R. conorii* IFA kit, whilst Laboratory 2 and 3 used the Focus Diagnostics IgG and IgM IFA kit. The expected results (obtained from using four (4) different assays, described above), and the results obtained by the participants, are summarised in Table 4.3. All participants reported using manufacturers' suggested protocols for the tests.

Overall agreement of results of 83% for IgG and 77% for IgM were achieved. Laboratories 2 and 3 reported false-positive IgG results for sample 2018/01-003, whilst all three (3) participants reported false-positive results for sample 2018/01-

010. No false-negative results were reported. For IgM, only laboratory 3 reported full agreement with expected results. Laboratories 1 and 2 collectively reported seven (7) false-positive results for IgM. Half of the IgM results reported by laboratory 1 were false positive.

All three (3) laboratories offered interpretation of negative and positive results in accordance with the kit manufacturers' recommendations. Importantly this included wording that indicated that negative results do not exclude the diagnosis of TBF. Two (2) of the laboratories indicated the importance of considering the testing of paired samples.

Table 4.3. Inter-laboratory comparison results for rickettsia IgG and IgM tests.

Sample reference number	Expected IgG result	Participant IFA IgG results			Agreement of results for all participants	Expected IgM result	Participant IFA IgM results			Agreement of results for all participants
		Lab 1*	Lab 2†	Lab 3†			Lab 1*	Lab 2†	Lab 3†	
2018/01/001	NEG	NEG	NEG	NEG	100 %	NEG	POS	NEG	NEG	67 %
2018/01/002	POS	POS	POS	POS	100 %	NEG	POS	NEG	NEG	67 %
2018/01/003	NEG	NEG	POS	POS	33 %	NEG	NEG	NEG	NEG	100 %
2018/01/004	POS	POS	POS	POS	100 %	NEG	POS	NEG	NEG	67 %
2018/01/005	NEG	NEG	NEG	NEG	100 %	NEG	NEG	NEG	NEG	100 %
2018/01/006	NEG	NEG	NEG	NEG	100 %	NEG	POS	NEG	NEG	67 %
2018/01/007	POS	POS	POS	POS	100 %	NEG	NEG	NEG	NEG	100 %
2018/01/008	NEG	NEG	NEG	NEG	100 %	NEG	NEG	POS	NEG	67 %
2018/01/009	POS	POS	POS	POS	100 %	NEG	POS	POS	NEG	33 %
2018/01/010	NEG	POS	POS	POS	0 %	NEG	NEG	NEG	NEG	100 %
Agreement of results per participant (%)		90 %	80 %	80 %	83 %		50 %	80 %	100 %	77 %

* Viracell IgG or IgM IFA kit

† Focus Diagnostics IgG and IgM IFA kit

4.4 DISCUSSION

TBF is a common disease in South Africa and although diagnosis is often based on clinical presentation alone, astute laboratory confirmation is recommended to ensure that patient management is well directed. At the time of this study, rickettsial serological testing was routinely available from five (5) pathology providers in South Africa of which three (3) participated in the ILC exercise. Although the IFA is currently regarded as a gold standard method for TBF diagnosis, the value of PCR testing on appropriate samples has been widely reported. Although not subject to ILC in this study, it was determined that PCR testing was available from three (3) laboratories in South Africa. It was noteworthy that PCR-based testing was not available in state-operated facilities. In addition, only one (1) of the laboratories recommended PCR as a supplement to serological testing in their interpretation of negative serology findings.

There was a good agreement of results for IgG testing across the panel and participants interpreted these results in line with the recommendation of the commercial kit providers and knowledge regarding cross-reactivity among rickettsial species, and the requirement of follow-up testing for confirmation. The latter is important in the light of known high seroprevalence indicated in previous studies in South Africa, in order to elucidate previous versus current infections. A deficiency of the ILC exercise was that the measured serological titres or optical densities were not included in reporting and laboratories were expected to only report their final interpretation of the results. This information could have been useful to investigate the calling of false-positive results, particularly for sample 2018/01-010. The latter sample tested consistently negative by the panel provider with the two (2) kits used by the participants, and also with additional two (2) commercial kits available at the time of the study.

The agreement of IgM results was less encouraging, with one (1) of the participants reporting false-positive results for half of the panel. This laboratory employed the Vircell IgG/IgM kit whilst the other two (2) participants used the kit supplied by Focus Diagnostics. Importantly, the samples tested repeatedly negative at the hands of the panel provider before and following the ILC exercise, using the Vircell kit but also the additional three (3) commercial kits for IgM detection. The participant indicated no deviation in the test protocol as recommended by the manufacturer, and included the initial IgG pre-absorbing step, which is a common reason for false positives with IgM serological assays. As IFA is a microscopy-based method, requiring subjective interpretation of visual observation, this may offer an explanation for the false-positive results (for example with the observation of background autofluorescence). To our knowledge at the time of this study, only one (1) comparative study looking at commercially available *Rickettsia* IFA kits had been published by Kantsø *et al.* in 2009, which focused on the comparison of Focus Diagnostic IgG IFA kits with the Fuller Laboratories IgG IFA kits, and reported 100% agreement between the two (2) kits was when results were dichotomized in accordance with manufacturers' cut-off values (Kantso et al., 2009). This is generally regarded as a disadvantage of this methodology. In response to the ILC results the laboratory in question followed up on the reporting of false positive IgM results and concluded that they have indeed been 'overcalling' background fluorescence, and have adjusted their protocol and training of staff accordingly. Recently (2021), a study was conducted by Fomda *et al.* to evaluate several serological assays for rickettsial disease diagnosis, and found that although the gold standard diagnostic test, IFA, has the highest sensitivity and specificity, requires a high-cost fluorescent microscope and an expert technical person to interpret the results (Fomda et al., 2021). Authors reported that ELISA is a better replacement for WF test in the diagnosis of rickettsial diseases attributed to its high sensitivity and specificity, its ease to perform, necessity for only low cost equipment and non-reliance on a highly skilled technical staff to perform the testing and interpret results. Additionally, a large number of samples can be batched and run simultaneously, thus making it a

favourable test in many hospital-based diagnostic laboratories (Fomda et al., 2021).

Interestingly, none of the participants considered the use of the commercial IgM ELISA assay, which offers the advantage of objective result interpretation. In addition, laboratory 2 reported two (2) false-positive results. The rate of false-positive IgM findings was worrying, as these would support the incorrect diagnosis of TBF and may detract from the appropriate management of cases. A limitation of this study was the non-availability of IgM-positive serum samples, and this deficiency should be addressed in future ILC exercises to offer a more comprehensive analysis of inter-laboratory performance.

The availability of proficiency testing panels for rickettsia serology globally is very limited, and given species diversity that varies geographically available panels may not represent the species of concern in a given region or country. Therefore, the continued measure of performance across laboratories using well-characterised, local population-derived clinical material is encouraged.

4.5 CONCLUSIONS

Currently there is no standardisation of laboratory diagnosis of TBF in South Africa. The consequence is variable reliability of test results and possibility a devaluation of the role of laboratory confirmation of the disease. As commercial kits are used for serological diagnosis, the importance of local population-based validation to gain better understanding of test sensitivity and specificity in the South African context cannot be understated. Expanding the use of PCR could be an important contribution for the investigation of suspected TBF cases, and again the use of validated methods subject to proficiency testing is encouraged.

To our knowledge, this is the first national ILC for rickettsial serology that has been conducted in South Africa. The value of continued ILC efforts for rickettsial serology, but also PCR, in South Africa is recognized and may contribute to the standardisation and improvement of the quality of laboratory investigations for TBF in the country.

CHAPTER 5 - SUSPECTED CASES OF CRIMEAN-CONGO HAEMORRHAGIC FEVER DIAGNOSED WITH TICK BITE FEVER IN SOUTH AFRICA, 2011-2018

5.1 INTRODUCTION

A large variety of pathogens including parasites, bacteria and viruses are transmitted by ticks, but it remains difficult to fully assess their impact since prevalence, distribution and burden of these neglected tropical diseases are not well known or understood in sub-Saharan Africa (Swei et al., 2020, Madison-Antenucci et al., 2020, Mwamuye et al., 2017, Chitanga et al., 2014, Halajian et al., 2018, Adelabu et al., 2019). There are at least twenty (20) Ixodid tick species in SA that feed on humans as incidental hosts (Horak et al., 2002, Chitanga et al., 2014). Several of these species are capable of transmitting various endemic TBD including, CCHF caused by Crimean-Congo haemorrhagic fever virus; ehrlichiosis caused by *Ehrlichia ruminantium*, *Ehrlichia canis* and *Anaplasma phagocytophilum*; babesiosis caused by *Babesia microti*; relapsing fever caused by *Borrelia duttonii* and rickettsioses caused by *Rickettsia africae*, *Rickettsia aeschlimannii* and *Rickettsia conorii* (Horak et al., 2002, Chitanga et al., 2014, Seo et al., 2018, Guo et al., 2019, Adelabu et al., 2020, Chiuya et al., 2020).

Crimean-Congo haemorrhagic fever has been reported in over 30 countries on three (3) continents (i.e. Africa, Asia, and Europe), and is primarily considered an occupational risk of the agricultural and livestock farming sectors, and the medical or veterinary sectors, particularly in rural settings (Chitanga et al., 2014, Chiuya et al., 2020). The CCHF virus belonging to the genus *Orthonairovirus* (family *Nairoviridae*, order *Bunyavirales*), within the realm *Riboviria* (a new megataxonomic taxon rank according to most recent classification based on the advances in viral genomic and metagenomic comparative analysis) (Portillo et al.,

2021), usually circulates in an enzootic tick-vertebrate-tick cycle and infects vertebrate hosts, including livestock (Chitanga et al., 2014, Kemp et al., 2014, Chiuya et al., 2020, Linthicum and Bailey, 1994). Despite the CCHF virus having been reportedly associated with several tick genera, CCHF foci are regularly associated with *Hyalomma* species ticks, specifically *Hyalomma rufipes* and *Hyalomma truncatum*, (Chitanga et al., 2014, Kemp et al., 2014, Linthicum and Bailey, 1994). In SA, CCHF has been reported since 1981. The disease is rarely reported in humans with a total of 205 cases confirmed between 1981-2019 with an overall mortality rate of 30% (Personal communication: J Weyer, NICD/NHLS). Human CCHF cases in SA are linked to tick bites or other tick exposures in more than two-thirds of cases (Kemp et al., 2014).

African tick bite fever has been increasingly reported as one (1) of the most common causes; second only to malaria; of systemic febrile illness in travellers returning from sub-Saharan Africa (Prado et al., 2017, Althaus et al., 2010, Cox and Visser, 2015, de Almeida et al., 2010). In SA, there are predominantly two (2) spotted fever rickettsioses that have been described, one (1) caused by *R. conorii* (i.e. boutonneuse fever-like TBF) and the other caused by *R. africae* (i.e. ATBF) (Caruso et al., 2014, Frean and Grayson, 2019). *Rickettsia conorii* was first reported as the causative agent of Boutonneuse fever (also known as MSF) in 1910 by Conor and Bruch, who described the bacterium as a strict intracellular organism transmitted to humans by the dog tick *Rhipicephalus sanguineus* (Caruso et al., 2014, Kemp et al., 2014). Patients infected with *R. conorii* present with fever, generalised maculopapular rash and an eschar or 'tache noire', a small necrotic skin lesion encircled by an inflammatory halo associated with *in situ* bacterial proliferation (Caruso et al., 2014). African tick bite fever was first described in the 1930s by Pijper, who characterised the infection as a rural and benign disease, rarely associated with cutaneous rashes. Pijper, through experimentation with infected animals, deduced that ATBF was caused by a species different to that responsible for boutonneuse fever. The isolate was however lost, and for the following six (6) decades, ATBF and boutonneuse fever

were both erroneously attributed to *R. conorii* and collectively termed TBF (Caruso et al., 2014). *Rickettsia africae* was later isolated and confirmed as a new species in 1992, where it was unarguably shown to be the etiologic agent of ATBF (Caruso et al., 2014). There have also been single case reports of other spotted fever rickettsial infections in humans in SA (i.e. *R. aeschlimannii* and *R. mongolotimonae*), however their contribution to the local burden of disease is unknown, since molecular diagnosis and genotyping are not routinely used (Frean and Grayson, 2019).

Tick bite fever usually presents as mild and self-limiting disease, with non-specific symptoms including, febrile illness, headaches, myalgia, lymph node swelling, and maculopapular eruption with inoculation eschars (Chmielewski et al., 2013, Frean and Grayson, 2019, Ehounoud et al., 2017). However, despite the majority of these cases being mild, some rickettsial infections present with severe outcomes for patients, where hospitalization with intensive care management is necessary and in some instances, patients die. Complicated and severe rickettsial diseases present with multi-organ involvement, encephalitis, confusion, or coma; pneumonia; deep vein thrombosis resulting in pulmonary embolism; bleeding; gangrene; hepato-renal failure; and even myocarditis (Frean and Grayson, 2019). In *R. conorii* TBF infections where diagnosis and treatment are delayed, disease may present, and mimic meningococcal septicaemia, other fulminant gram-negative septicaemia with disseminated intravascular coagulopathy, or as a viral haemorrhagic fever such as CCHF (Frean and Grayson, 2019). TBF in SA has a documented case fatality rate of 3%, and this number could be higher for cases that are missed or miss-diagnosed (Kemp et al., 2014).

This study describes a case series of patients presenting with suspected CCHF but diagnosed with TBF in SA between 2011-2018. The clinical aspects and genetic characterization of *Rickettsia* species involved in these cases are herein presented.

5.2 MATERIALS AND METHODS

5.2.1 CASE SELECTION

The CEZPD of the NICD/NHLS is the reference laboratory for investigation of viral haemorrhagic fevers, including CCHF, in South Africa. A retrospective document review (including laboratory sample registers and case submission documents) was conducted to identify cases that were submitted for diagnostic investigation for CCHF, but were found to be PCR positive for *Rickettsia* during differential diagnostic investigations conducted at the NICD/NHLS between 2011-2018, for inclusion into this study. Further inclusion criteria were: sufficient residual sample volume available for further genetic characterization and the availability of archived case histories at the NICD/NHLS.

5.2.2 ETHICS STATEMENT

Ethical clearance for this study was obtained prior to its commencement through the University of Pretoria Human Research Ethics Committee (UP HREC) (protocol reference number: 222/2017) (Appendix 3.3). Although the case histories were accessed, the personal information of the patients were confidentially managed. No patient personal information will be presented herein.

5.2.3 DATA EXTRACTION

Data was extracted from the submission request forms, laboratory reports and clinical notes archived at the NICD/NHLS. The following data was recorded to reveal possible factors relating to the severe outcomes of disease: patient demographics (i.e. gender, age), exposure history (i.e. history of tick bite or other exposure, date of exposure, geographical location of exposure), clinical presentation (i.e. date of onset of illness; date of hospitalization; presence of an eschar/s, and eschar site (if applicable); presence of rash and type of rash, history of fever and other symptoms, clinical outcome, i.e. survive or perish), pathological

findings (including platelet count, liver enzymes level, white cell count, c-reactive protein count) and clinical management (i.e. treatment provided). The data was summarized in a Microsoft® Excel worksheet and is presented Table 5.3 in section 5.3.3.

5.2.4 CASE DATA ANALYSIS

The data were collected in a Microsoft® Excel spreadsheet. Basic descriptive statistics were summarized by using Microsoft® Excel formula functions.

5.2.5 DNA EXTRACTIONS

DNA was extracted from the clinical samples (i.e. blood or eschar swabs) using the QIAamp Viral RNA kit (Qiagen, Hilden, Germany) on the QIAcube automated extractor (Qiagen, Hilden, Germany). The protocol was modified to allow for an 'off-board' manual lysis to occur prior to the continuation of the manufacturer's protocol. Briefly, 140 µL of homogenized sample was added to 560 µL of prepared Buffer AVL containing carrier RNA in a 1.5 mL microcentrifuge tube and thoroughly homogenized by pulse-vortexing for 15 s, followed by a 10 min incubation at room temperature. The mixture was then briefly centrifuged to remove droplets from the inside of the lid, followed by the addition of 560 µL of ethanol (96 to 100%) to the mixture. The mixture was then briefly homogenized by pulse-vortexing for another 15 s and subjected to a short (5 to 15 s) spin to remove droplets from inside the lid. At this point the QIAcube DNA extraction robot was set up for the number of samples that were to be extracted (to a maximum of 12 per run) and the remainder of the extraction steps were carried out by the extractor with a final elution of 60 µL in buffer AVE.

5.2.6 PCR ASSAYS

The PCR reactions were performed using the Expand™ High Fidelity PCR System (Roche Diagnostics GmbH, Mannheim, Germany) in accordance with manufacturer protocols and in-house optimisation using the Taguchi method (Cobb and Clarkson, 1994) (results not shown). The PCR was optimised using DNA extracted from three (3) American Type Culture Collection (ATCC) isolates, i.e. *R. akari* (VR612), *R. akari* (VR148-2) and *R. raoulti* (DNS14). At least one (1) of the ATCC culture DNA samples were included into each assay thereafter to serve as a positive control and nuclease free water was used as the non-template control. Primers were designed in-house and targeted a 333 bp portion of the SFG rickettsial citrate synthase (*gltA*) gene:

RickF 5'-GACGGTATACCCATACGTTTATAG-3' and
RickR 5'-TAATGAAGCGGTAATAAATATG-3'.

Each reaction contained; 5 µL of Expand High Fidelity Buffer (10×) with 15 mM MgCl₂, 200 µM of each dNTP, 0.3 µM of relevant primers, 2.6 U Expand High Fidelity Enzyme mix, 10 µL of extracted DNA and nuclease-free water to a final volume of 50 µL. Reactions were amplified on a Veriti® Thermal cycler (Applied Biosystems, Thermo Fisher Scientific) under the following conditions: initial denaturation step of 94°C for 2 min followed by 40 cycles of denaturation at 94°C for 20 sec; annealing at 47°C for 30 sec, extension at 68°C for 30-60 sec; and a final extension at 68°C for 5 min and hold at 4°C (infinite). PCR products were electrophoresed at 100V for 45 – 60 min on 1.2% (w/v) TBE-agarose gels, stained with GelRed (Biotium, Inc., USA) and size fractionated using a 100 bp molecular weight marker (mwm) (Bioline, UK). Amplicons of the expected size, i.e. 333 bp, were thereafter cleaned up and sequenced as described in 5.2.7 and 5.2.8 below.

5.2.7 PCR AMPLICON PURIFICATION

Amplicons were purified using the Wizard® SV Gel and PCR Clean-up System (Promega, Madison, WI, USA) as per manufacturer protocol. Briefly, an equal volume of binding buffer was applied to the PCR product and the mixture was homogenized by pipette mixing. The full volume of the mixture was then applied directly onto the filter portion of the spin column and incubated for 1-3 min at room temperature, followed by centrifugation at maximum speed (~13 000g) for 1 min. The flow-through was discarded and 700 µL of wash buffer was applied to the spin column. This was spun at maximum speed for 1 min and the flow-through was then also discarded. A final wash step was done by applying 500 µL of the wash buffer to the column and again spinning at maximum speed for 5 min. The flow-through was discarded and the column was 'dry spun' for 1 min at maximum speed to remove any remaining ethanol. The column was then placed into a sterile, nuclease-free micro-tube, followed by the addition of 40 µL of nuclease-free water. This was allowed to incubate at room temperature for one (1) to three (3) min and then centrifuged as before.

5.2.8 SANGER SEQUENCING

Purified amplicons were measured for concentration (in ng/µL), purity (i.e. 260/280¹ ratio > 1.8) and quality (i.e. 260/230² ratio in the range of 2.0-2.2) using the NanoDrop 2000 or 2000c (Thermo Scientific™) spectrophotometers. The purified amplicons of appropriate concentration (i.e. a minimum concentration of 10 ng/µL) and satisfactory purity (i.e. ratios greater than 1.8) were then subjected to sequencing. Cycle sequencing reactions were performed using BigDye™

¹ The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as 'pure' for DNA; a ratio of ~2.0 is generally accepted as 'pure' for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

² This ratio is used as a secondary measure of nucleic acid purity. The 260/230 values for 'pure' nucleic acid are often higher than the respective 260/280 values. Expected 260/230 values are commonly in the range of 2.0-2.2. If the ratio is appreciably lower than expected, it may indicate the presence of contaminants which absorb at 230nm.

Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Scientific, Carlsbad, CA, USA) as recommended by the manufacturer and using 3.2 μ M RickF or RickF per reaction. Sequencing reactions were subjected to a clean-up step to remove any free and labelled dNTP's, to desalt and to remove traces of phenol or buffer salts in preparation for sequencing. The clean-up was carried out using the Centri-sep columns (Princeton Separations, Adelphia, NJ, USA) as per manufacturer protocols. Sequencing reactions were then transferred to 0.2 mL microtubes, and allowed to desiccate by incubating at 95°C on a heating block for 10 to 25 min (depending on volume). Once the reactions were completely dehydrated, 10 μ L of Hi-Di™ Formamide (Applied Biosystems, Thermo Fisher Scientific, USA) was applied to the desiccated sequencing reactions, and these were then returned to the thermocycler for 3 min at 95°C to allow for denaturation to occur. The formamide mixtures were then transferred to a MicroAmp Optical plate (Applied Biosystems, Thermo Fisher Scientific, USA) to be loaded onto the sequencer. Sequencing reactions were resolved on an ABI 3500 XL DNA Sequencer (50 cm capillaries) with POP-7™ separation matrix (Applied Biosystems, Thermo Fisher Scientific, USA).

5.2.9 SEQUENCE DATA ANALYSIS

The sequence data obtained was analysed using BioEdit version 7.2.5 (BioEdit Sequence Alignment Editor© 1997-2013 Tom Hall) and sequences were compared with those on GenBank using the NCBI BLAST 2.0 tool (National Centre for Biotechnology Information, Bethesda, MA, U.S.A.; <http://www.ncbi.nlm.nih.gov/blast/>) for homology searching. A multiple-sequence ClustalW alignment was performed, aligning the severe case consensus sequences with other SFG Rickettsial sequences obtained from GenBank (Ehlers et al., 2020). Sequence accession numbers are tabulated below (Table 5.1). Sequences were edited at the 5' and 3' ends so that sequence lengths trimmed that of the longest sequence common to all species compared (Fournier et al., 2003).

Table 5.1 Genbank accession numbers for sequences used in phylogenetic analyses.

Accession number	Species and strain	Accession number	Species and strain
U59733	<i>Rickettsia africae</i> ESF-5	U59724	<i>Rickettsia japonica</i> YM
MG515012	<i>Rickettsia africae</i> strain PELE	U59722	<i>Rickettsia aeschlimannii</i> . MC16
HQ335126	<i>Rickettsia africae</i> strain EgyRickHd-Qalet El-Nakhl	U59721	<i>Rickettsia rhipicephali</i> 3-7-6
HM536211	<i>Rickettsia africae</i> strain PoHuR20831dt	U74756	<i>Rickettsia montana</i>
HM050288	<i>Rickettsia africae</i>	U59734	<i>Rickettsia sibirica</i>
U59730	<i>Rickettsia conorii</i> Seven	U59719	<i>Rickettsia massiliae</i> Mtu 1
U59732	<i>Rickettsia parkeri</i> Maculatum 20	U59718	<i>Rickettsia australis</i> Phillips
HM050292	<i>Rickettsia conorii</i>	U59717	<i>Rickettsia akari</i> MK (Kaplan)
MK295621	<i>Rickettsia conorii</i> isolate R5	U59726	<i>Rickettsia honei</i> NTT-118
MF002509	<i>Rickettsia conorii</i> isolate Xinjiang-PS	U59725	<i>Rickettsia slovaca</i> N.A. 13-B
KY069261	<i>Rickettsia conorii</i> isolate SHZ	JN375498	<i>Rickettsia felis</i> isolate LI16
JN182786	<i>Rickettsia conorii</i> clone 68	AF210692	<i>Rickettsia</i> sp. California 2
HM152564	<i>Rickettsia conorii</i> strain LHR BR		

Phylogenetic analyses were performed to infer evolutionary history, using the Maximum Likelihood, the Neighbor-Joining, and the Maximum Parsimony (MP) methods as implemented by the Molecular Evolutionary Genetics Analysis version 7.0 (MEGA7) software package (Kumar et al., 2016). The Maximum Likelihood method, based on the Tamura-Nei model (Tamura and Nei, 1993), demonstrated the tree with the highest log likelihood. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The frequency with which the associated taxa clustered together out of 1,000 bootstrap replicates, were shown as percentages adjacent the branches. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The second method, the Neighbor-Joining method (Saitou and Nei, 1987) also presented the optimal tree, with the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 bootstrap replicates) presented adjacent to the branches (Felsenstein, 1985a). The final tree

constructed to infer evolutionary history was the MP method, where the most parsimonious tree (Tree #1 out of 10) was presented. This tree also demonstrated the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) (Felsenstein, 1985a) and was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (pg. 126 in Nei and Kumar, 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). This tree was also drawn to scale, with branch lengths calculated using the average pathway method (Nei and Kumar, 2000) and are in the units of the number of changes over the whole sequence. All three (3) trees were rooted using the transitional group *R. felis* (GenBank accession number: JN375498.1) as the outgroup.

5.3 RESULTS

5.3.1 CASES

A total of 350 samples (43.75 on average per year) were submitted as suspected CCHF patients from 2011 to 2018 at the NICD/NHLS (Table 5.2). A total of the 313 samples tested negative for CCHF during this period. During differential diagnostic investigations, approximately 27% (87/313) of the samples that tested negative for CCHF, were tested for TBF by *Rickettsia* PCR/or *Rickettsia* IgG/IgM serology. A total of 31 of these samples tested positive (31/87, 36%) by *Rickettsia* PCR and/or IgG/IgM serology. For the purpose of this study, all serological positives were excluded, with only PCR positives of sufficient quantity for additional PCR assays and for which clinical histories were available were included in this study. To further elaborate, of the 32 CCHF tests performed in 2011 for example, 30 were found to be negative. Of the 30 CCHF-negative samples, *Rickettsia* diagnostic assays were performed on seven (7) samples (either by serological analysis or by molecular assay or both). Three (3) of the seven (7) samples tested for *Rickettsia* were found to be positive, but only one (1) of the three (3) *Rickettsia*-positive samples met the additional selection criteria as described in section 5.2.1. Ultimately, only five (5)

cases of the total 31 *Rickettsia*-positive cases described in Table 5.2 could be included in the study, having met all inclusion criteria described.

Table 5.2. CCHF and *Rickettsia* patient requests tested at SVPRL/CEZPD, 2011-2018.

YEAR	CCHF SAMPLES TESTED		TOTAL CCHF TESTS/YEAR	RICKETTSIA DIFFERENTIAL TESTS CONDUCTED		TOTAL TBF TESTS FROM CCHF –VE SAMPLES TESTED/YEAR
	Positive	Negative		Positive	Negative	
2011	2	30	32	3	4	7
2012	0	24	24	2	4	6
2013	10	38	48	2	6	8
2014	5	49	54	9	11	20
2015	1	24	25	7	8	15
2016	1	42	43	3	13	16
2017	14	64	78	2	5	7
2018	4	42	46	3	5	8
TOTAL	37	313	350	31	56	87

5.3.2 Epidemiological features

5.3.2.1 Demographics of the cases

A total of five (5) of the 31 (see Table 5.3) *Rickettsia*-positive patient samples met the inclusion criteria for this study, two (2) were from male patients and three (3) were from female patients. All patients were adults aged between 28 – 70 years, with the median age being 37.

5.3.2.2 Exposure histories (including geographical distribution)

Four (4) of the five (5) patients did not report a history of travel that could account for a tick exposure, however all the patients were exposed to ticks in rural settings. Only one (1) of the patients reported having travelled to the Vaal River, Free State Province, whereupon the tick exposure was thought to have occurred. Two (2) of

the patients were infected in Free State (FS) Province, one (1) at the Vaal river (as mentioned above) and the other on a small-holding or plot in the rural outskirts of Bloemfontein; one (1) on a cattle farm in Kimberly, in the Northern Cape (NC) Province; one (1) from rural Vryburg in the North-West (NW) Province; and one (1) from Heidelberg in Gauteng Province (GP). Tick exposures were recalled for four (4) of the five (5) patients, with eschars (such as the example provided in Figure 5.2) being observed on these four (4) patients.

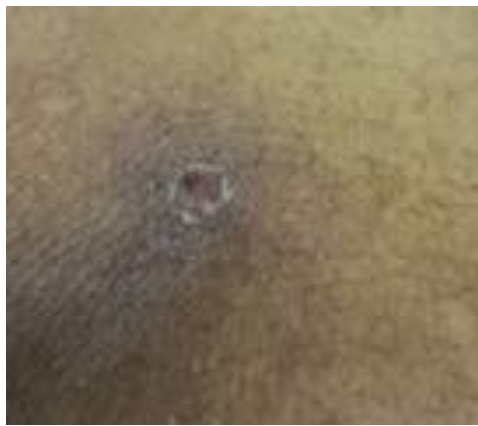


*Figure 5.1. Healed eschar from a fatal case of TBF in 2018. This picture is taken from another *R. conorii* case that was sent to the CEZPD laboratory as a query CCHF. It was unfortunately not included in the cases described in this study due to insufficient material.*

5.3.2.3 Clinical presentation (including outcome) and management

All the patients of this study were severely ill, hospitalized and receiving intravenous antibiotic treatment in intensive care units (ICU). Patient samples were all received as VHF referrals. Rashes (maculopapular, vasculitis and/or petechial) were observed on three (3) of the patients and all five (5) presented with fever. Other symptoms observed in the patients included joint and body pain, confusion, low blood pressure, chills, malaise, headache, nausea, vomiting, diarrhoea, dehydration, reduced consciousness, conjunctivitis, bleeding gums, jaundice, epistaxis, convulsions, low urine output, and/or blackened toes. The cases were all treated with antibiotics, with only one of the patients being treated with the

recommended antibiotic for treatment of rickettsiosis, namely doxycycline. Unfortunately, this patient also succumbed to the infection and died.

One (1) of the patients, a 70-year-old male, was found comatose and dehydrated before being admitted to ICU. He reportedly complained of body and joint pain for eight (8) days preceding hospitalization, and was febrile with a maculopapular rash and eschar on his abdomen. The patient was treated with augmentin, anti-inflammatories for a week which unfortunately had no effect on his recovery. He briefly experienced confusion when he regained consciousness, but later demised. The second patient included in this study was a 28-year old, confused and delirious male who was admitted to ICU following a fishing trip whereupon the patient had reportedly experienced multiple tick bites approximately seven days prior to his onset of illness and had multiple eschars on his body. He presented with a fever and a vasculitic rash on the palms of his hands but not on the soles of his feet and following a PCR positive for *Rickettsia*, was treated with intravenous ciprofloxacin and recovered. The third patient, a 46-year old female, presented with fever and petechial rash was admitted to ICU. She had no history of tick exposure that could be recalled and no visible eschars observed upon examination. The fourth patient was a 52-year old female who was admitted to ICU four (4) days after onset of illness. She had recalled being bitten by a tick and upon examination, an eschar was observed on the left, lower leg. The patient was febrile and complained of chills and malaise and was initially treated with meropenem, then later received a combination therapy of doxycycline and moxifloxacin two (2) days after hospital admission. The patient consequently demised despite having received the recommended treatment for TBF. The fifth and final patient included in this study, was a 34-year old female who was admitted to ICU with fever and a maculopapular rash on her body, including the palms of her hands. Tick exposure had reportedly occurred seven (7) days prior to onset of illness and two (2) eschars were observed on the patient's neck. The patient was hypotensive, and bleeding, had low urine output, suffered convulsions and her toes had reportedly gone black. Due to the bleeding symptom, this case was highly suspected to be CCHF/VHF, but was

found to be *Rickettsia*-positive. This patient was treated with multiple antibiotics including tigecycline and ciprofloxacin but the patient subsequently demised.

5.3.2.4 Pathological findings

All five (5) of the patients presented with low platelet counts ranging from 20-45 mcL, far below the lower normal threshold (i.e. 150-400 mcL). The 70-year-old patient had a very low (35-29 mcL) platelet count, liver enzymes ALT of 109 U/L and AST of 274 U/L, the low to normal (3.55-7.73 cells/L) white blood cell (WBC) count, and the C-Reactive protein (CRP) was far above normal (normal: < 10 mg/L) at between 202-240 mg/L. The 28-year-old patient presented with a low platelet count (43 mcL), liver enzyme ratio (alanine transaminase (ALT) and aspartate transaminase (AST)) was AST/ALT 250 U/L, and elevated WBC and CRP counts at 12.68 cells/L and 327 mg/L respectively. The 46-year-old female patient presented with a low blood pressure, low platelet count (45 mcL) and liver enzymes ALT/AST were >200 U/L. Unfortunately, the WBC and CRP counts were not available, and information regarding treatment administered and patient outcome were also not available for this patient. The 52-year-old patient's platelet count was very low (20 mcL), liver enzymes were AST of 253 U/L / ALT of 84 U/L, her CRP count was elevated (309 mg/L). She was immunocompromised (HIV +ve) with a CD4 count of 136. Her WBC count was normal (4.2 cells/L). The 34-year old female patient's platelet count was also low (38 mcL) and her WBC count was extremely high 3183 cells/L. No liver enzyme or CRP results were available to report.

5.3.3 *gltA* PCRs AND SEQUENCING RESULTS

Sequencing of the citrate synthase (*gltA*) gene was conducted for the all cases described previously. All of the phylogenetic tree topologies obtained using three (3) algorithms (i.e. Maximum Likelihood, the Neighbor-Joining, and the Maximum Parsimony) produced similar trees, and are shown in Figure 5.2, 5.3, and 5.4. The trees inferred using *gltA* partial sequences that the severe cases grouped as a

single distinct clade, closest to but diverging from the *R. conorii* sequences to which they were compared with bootstrap values of 63 % for each of the methods used. The sequences were comprised of 316 bp that were 100 % similar to each other.

Table 5.3. Patient metadata presenting with severe rickettsioses referred to the CEZPD/NICD for CCHF investigations, 2011-2018.

Patient	1 (244/11)	2 (339/14)	3 (296/15)	4 (370/15)	5 (492/18)
Gender	M	M	F	F	F
Age	70	28	46	52	34
Province	NCP	FS	FS	NW	GP
Type of location	Rural	Rural	Rural	Rural	Rural
Travel history	N	Y, fishing	N	N	N
Tick-bite history	Y	Y	N	Y	Y
Hospitalization	ICU	ICU	ICU	ICU	ICU
Sample tested	Blood	Eschar swab	Blood	Blood	Blood
Eschar	Y	Y	N OBS	Y	Y
Rash	Y	Y	Y	N	Y
Type of rash	Maculopapular	Vasculitic	Petechial	N/A	Maculopapular
Fever	Y	Y	Y	Y	Y
Body/joint pain	Y	N	N	N	N
Confusion/delirium	Y	Y	N	N	N
Reduced consciousness	Y	N	N	N	N
Convulsions	N	N	N	N	Y
Hypotensive	N	N	Y	N	Y
Bleeding/epistaxis	N	N	N	N	Y
Gangrene	N	N	N	N	Y
Malaise	N	N	N	Y	N
Chills	N	N	N	Y	N
Dehydrated	Y	N	N	N	N
Low urine output	N	N	N	N	Y
Platelets (mCL)	L 35-29	L 43	L 45	L 20	L 38

(normal: 150-450 mcl)					
Liver enzymes U/L	ALT 109; AST 274	AST/ALT 250	ALTS/AST >200	AST 253; ALT 84	UKN
White blood cells (WBC) (normal: 4.0-11.0 cells/L)	3.55-7.73	H 12.68	UKN	4.2	3.1 – 8.3
C-Reactive protein (CRP) (normal: <10 mg/L)	H 202-240	H 327	UKN	H 309	UKN
Treatment	Co-amoxiclav, anti-inflammatory	IV Ciprofloxacin	UKN	Meropenem, Doxycycline plus Moxifloxacin	Multiple antibiotics, tigecycline, ciprofloxacin
Outcome	Died	Recovered	UKN	Died	Died

Abbreviations: M, male; F, female; NCP, Northern Cape Province; FS, Free State Province; NW, North-West Province; EC, Eastern Cape Province; WC, Western Cape Province; GP, Gauteng Province; N, no; Y, yes; UKN, unknown; ICU, Intensive care unit; IV, Intravenous; N OBS, none observed; L, Low; H, High; ALT, alanine transaminase; AST, aspartate transaminase.

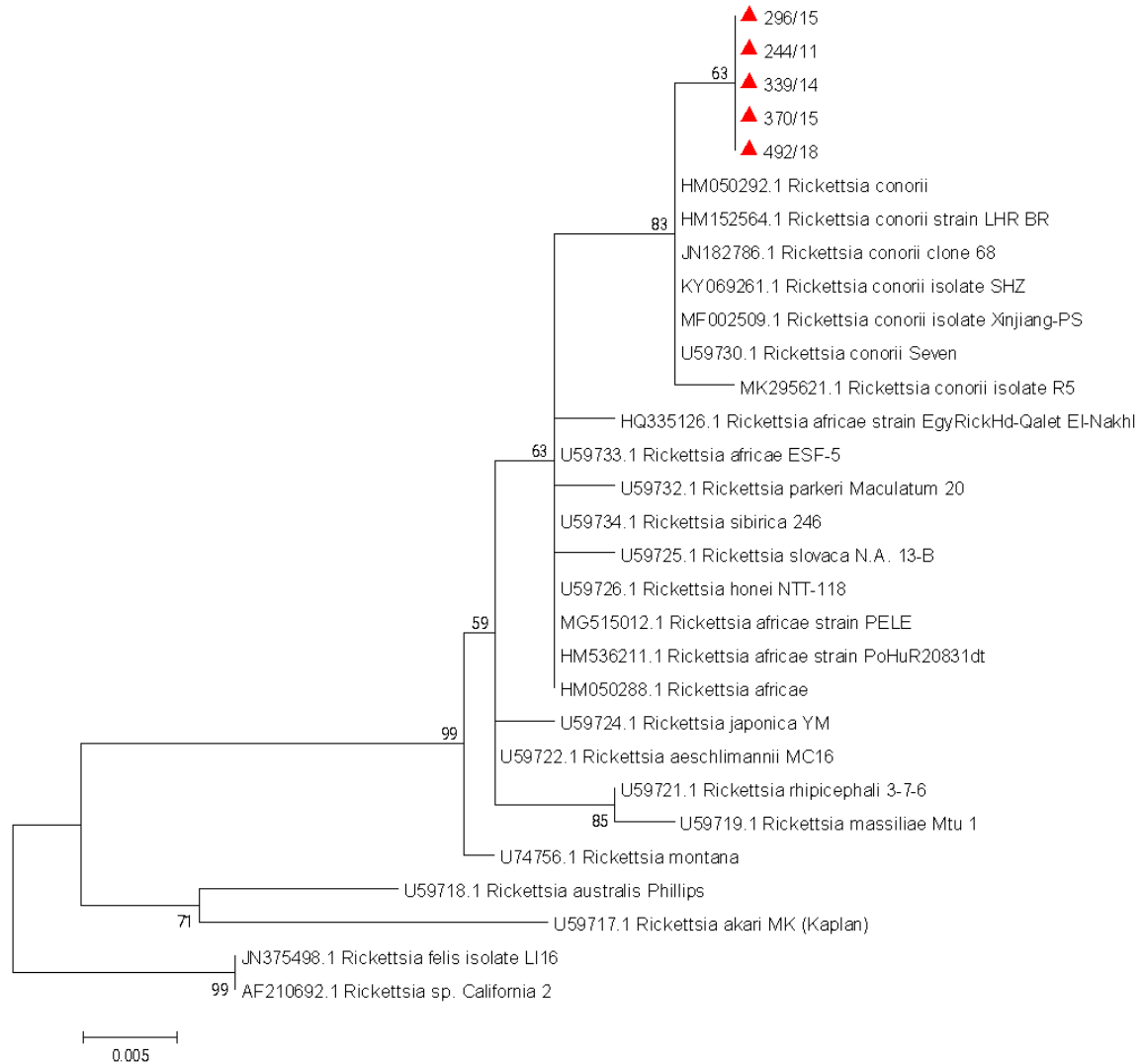


Figure 5.2. Molecular Phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-644.2135) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 30 nucleotide sequences. There were a total of 316 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

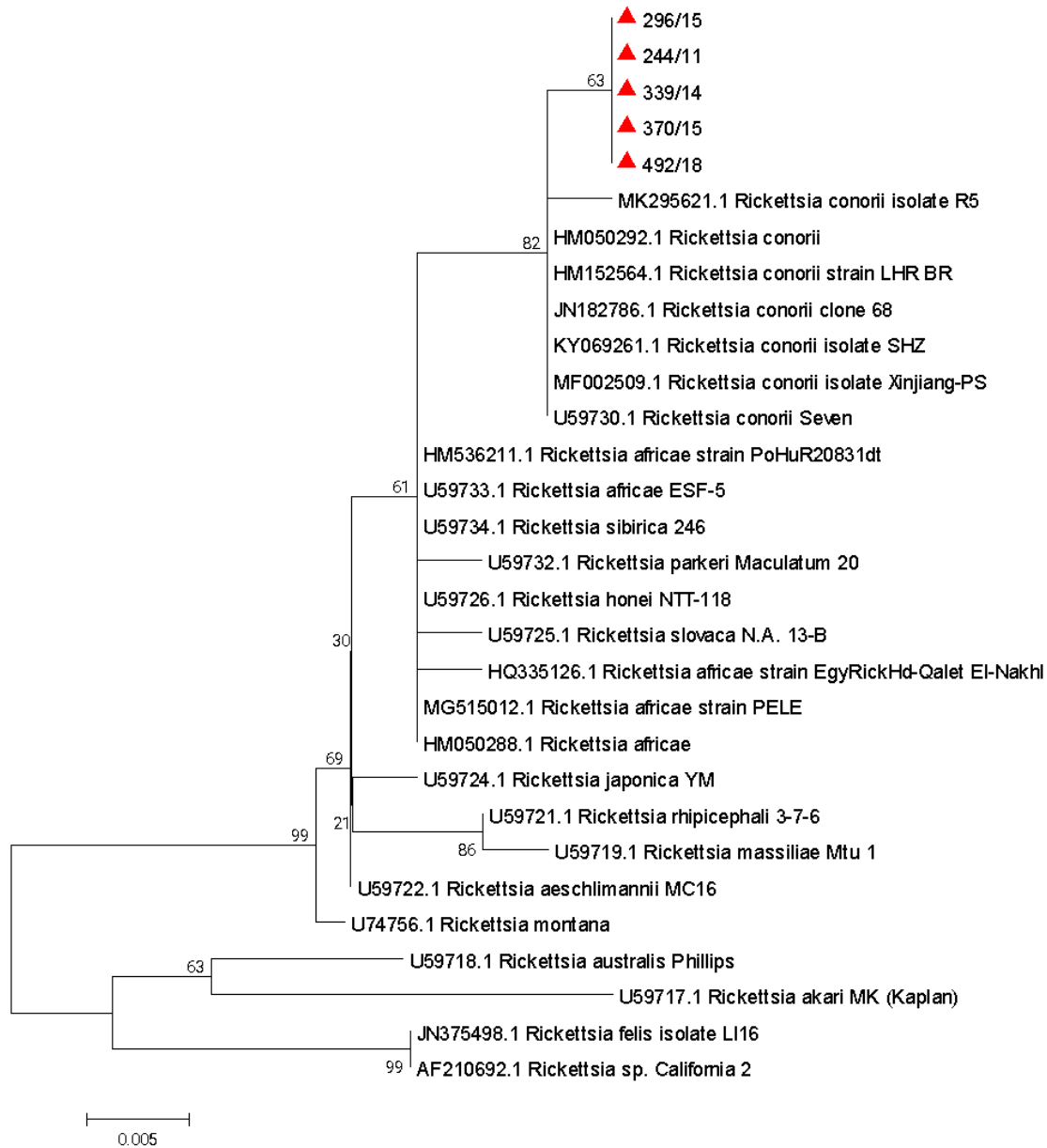


Figure 5.3. Evolutionary relationships of taxa. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.11014696 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985a). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura-Nei method (Tamura and Nei, 1993) and are in the units of the number of base substitutions per site. The analysis involved 30 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 316 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

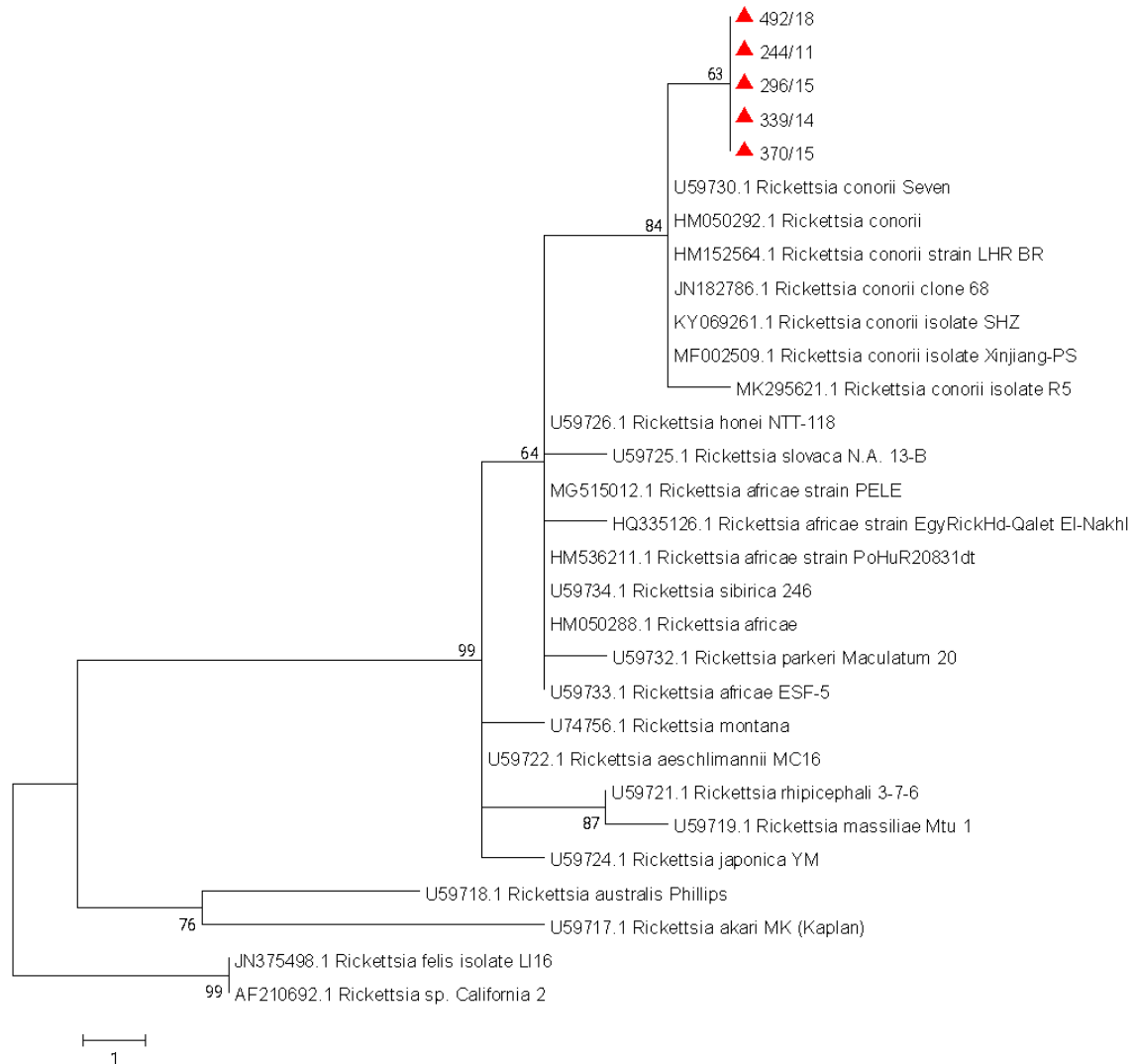


Figure 5.4. Maximum Parsimony analysis of taxa. The evolutionary history was inferred using the Maximum Parsimony method. Tree #1 out of 10 most parsimonious trees (length = 35) is shown. The consistency index is (0.909091), the retention index is (0.968254), and the composite index is 0.912925 (0.880231) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985a). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (pg. 126 in (Nei and Kumar, 2000)) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The tree is drawn to scale, with branch lengths calculated using the average pathway method [see pg. 132 in (Nei and Kumar, 2000)] and are in the units of the number of changes over the whole sequence. The analysis involved 30 nucleotide sequences. There were a total of 316 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

5.4 DISCUSSION

Tick bite fever is an important differential diagnosis for CCHF (Blumenthal et al., 2021) with geographic distribution, primary vectors and symptom presentations overlapping for both CCHF and severe TBF disease (de Almeida et al., 2010). In South Africa, rickettsial infection is generally attributed to one (1) of two (2) *Rickettsia* species, *R. africae* and *R. conorii*, the latter associated with the more severe clinical presentation and potentially fatal outcomes (Blumenthal et al., 2021). The clinical picture of a *R. conorii* TBF patient may be so severe that the attending clinician fails to anticipate *Rickettsia* as the cause of the illness and therefore affecting the timeous initiation of appropriate treatment regimens. Limited epidemiological data on tick-borne infections, as well as limited diagnostic capacity at the point of care facilities, further compound the problem of failure to anticipate TBFs (Kemp et al., 2014). Early infection laboratory confirmation is generally challenging due to low sensitivity of both PCR and serology techniques (Kemp et al., 2014, Biggs et al., 2016). The rickettsial infections described in this study, as with others reported elsewhere, were only discovered during differential diagnostic work-up being carried out in a reference laboratory employing a process of elimination of diseases like CCHF, dengue, yellow fever and leptospirosis (de Almeida et al., 2010). African TBF is considered an emerging disease for many travelers returning from endemic countries such as South Africa, Botswana and Zimbabwe, and more recently the Caribbean islands, where the disease is most often reported (Katswara and Mukaratirwa, 2021). Diagnosis is often challenging within endemic countries and areas where rickettsioses are often misdiagnosed as malaria, typhoid, and acute febrile disease, and it stands to reason that this would be more so problematic in non-endemic countries and areas not used to seeing a large number of ATBF cases (Armitano et al., 2018, Katswara and Mukaratirwa, 2021). There are many reports of ATBF in returning travelers, some with serious conditions such as myocarditis and pericarditis reported in a Swiss paper where two (2) patients holidaying in South Africa returned home and developed symptoms consistent with ATBF which later developed into more serious disease involving the heart (Bellini et al., 2005).

Bacterial vector-borne diseases are rarely reported in Africa, but it is thought that the real incidence rate may be as high as 13 % of all the febrile, exanthematic cases (Ehounoud et al., 2017). Other studies have reported TBF seroprevalences of up to 70 % in agricultural areas

where vectors and farming communities coexist (Frean and Grayson, 2019). Nevertheless, very few clinical cases of TBF have been reported in rural populations, seemingly due to the mild and non-specific clinical manifestations (Frean and Grayson, 2019) despite the vast majority of the South African population living in rural areas (Katswara and Mukaratirwa, 2021). The poorly resourced livestock farmers are often in direct and prolonged contact with domestic livestock and their ectoparasites (like ticks) thereby increasing the opportunity for transmission of zoonoses (Katswara and Mukaratirwa, 2021). Rickettsial diseases are common in southern Africa, with *R. africae* (the causative agent of ATBF), reportedly the most common of all the SFG rickettsiosis in terms of both seroprevalence and incidence (Fournier et al., 2009, Fournier et al., 2002, Gonzalez et al., 2012, Harrison et al., 2016, Katswara and Mukaratirwa, 2021). The second most prevalent SFG rickettsia in Africa, *R. conorii*, in contrast with *R. africae* exhibits a lower prevalence in ticks, and a sporadic occurrence with higher virulence in humans (Fournier et al., 2009, Fournier et al., 2002). *Rickettsia conorii* has been implicated as the causative agent for the severe outcomes of all five (5) cases described in this study. In each case, the clinical presentation was so severe with symptoms often seen in CCHF patients, that the attending clinicians suspected CCHF to be the cause of the disease. The non-specificity of symptoms and severe clinical presentation often misleads clinicians from anticipating *Rickettsia* as the etiological agents of the patient's disease (Kemp et al., 2014). Rickettsioses share a clinical picture that is consistent with a wide range of febrile illnesses, i.e. fever, myalgia and headache. Furthermore, patients presenting with flu-like symptoms often underestimate the condition or pass it off as 'flu' and do not seek medical evaluation at early onset, supporting the hypothesis that TBF is underreported in endemic regions (Kemp et al., 2014, Frean and Grayson, 2019).

It is imperative for clinicians to consider rickettsial infections for patients presenting with severe yet, non-specific symptomologies if the patient is to receive the correct treatment in time. The most vital aspect to timely and effective treatment of a TBF, is clinical recognition (Blanton, 2019). Medical practitioners should enquire about a patient's travel and exposure history, particularly regarding potential exposures to vectors, such as ticks, fleas, lice, and mites. Clinicians should familiarize themselves with the signs of tick exposures, so that they can recognise bites from these arthropods since these bites are often missed due to their small size,

and inconspicuous locations on the body such as hairlines, groin areas, behind knees and armpits (Blanton, 2019, Kemp et al., 2014). Often times, tick exposures are not recalled, particularly if bitten by ‘pepper ticks’, i.e. larval and nymph stage ticks. These immature stage ticks are also capable of transmitting *Rickettsias* since the infection is transmitted trans-stadially (between lifecycle stages) in the tick vectors. In addition, tick bites do not usually cause pain due to the production of anti-inflammatory substances within tick saliva (Blanton, 2019, Kemp et al., 2014). Clinicians are therefore advised to look for eschars as a possible clue for TBD and to check hidden areas around the head, neck and groin, since bite marks are not always in obvious or visible areas (Frean and Grayson, 2019). Clinicians should also look for healing eschars or scabs as shown in Figure 5.2. ATBF is often diagnosed based on the clinical presentation, particularly when the patient presents with fever and one or multiple eschars (Harrison et al., 2016).

Empiric treatment with an effective antibiotic should promptly be administered to patients who are suspected or reasonably suspected of having TBF. Many commonly prescribed antibiotics (e.g., penicillins, cephalosporins, and sulphonamides, such as trimethoprim-sulfamethoxazole) have little to no effect on rickettsiae (Blanton, 2019). Rickettsiae are generally most responsive to treatment with doxycycline, as soon after onset of symptoms (fever and sensitive, swollen lymph node/s) as is possible in order to prevent the infection from developing into more severe disease (Gudiol et al., 1989, Kemp et al., 2014, Frean and Grayson, 2019). Often, clinicians will need to initiate treatment without serological confirmation since *Rickettsia* infections can take up to 28 days for antibodies to reach detectable levels – particularly for *R. africae*. Furthermore, cross-reactivity between anti-*conorii* and anti-*africae* antibodies is common but the treatment is the same for both infections, therefore delaying treatment is ill-advised (Frean and Grayson, 2019). In this study, four (4) of the five (5) patients were known to have received antibiotic treatment, but little is known about the timeline from onset of illness to administration of treatment. Only one (1) of the patients is known to have recovered, and interestingly the patient was treated with intravenous ciprofloxacin. Although this is not the routinely recommended treatment option for rickettsial diseases, studies conducted as early as 1989 presented ciprofloxacin as a viable option for treatment of *R. conorii* infections, albeit it taking longer for the

defervescence of the patient (Gudiol et al., 1989, Jabarit-Aldighieri et al., 1992). The patient's response to the treatment can unfortunately not always be predicted and one (1) treatment may not be equally effective for all, as was observed in this study where one (1) patient recovered with the ciprofloxacin treatment regime, and another perished. The latter did, however, receive a number of other antibiotics as well, so it is unclear whether the ciprofloxacin was just started too late for the patient to recover, or whether the patient had compounding conditions that rendered the treatment ineffective in preserving her life. Of the five (5) cases presented, only one (1) was reportedly treated with doxycycline and this patient was treated with several other antibiotics as well, including meropenem, and moxifloxacin in combination with doxycycline, so it is unclear whether the recommended treatment, i.e. doxycycline, was just started too late, was administered at an ineffectual dosage or whether there were other confounding complications (i.e. her immunocompromised state) that could have lead to the patient's ultimate demise. In these cases presented, it is hypothesized that antibiotic treatment would likely have been initiated late, since all the specimens arrived at the laboratory as query CCHF, a disease for which the recommended treatment is supportive care, and in some cases, treatment with the anti-viral ribavirin (Saleem et al., 2020).

A challenge of this study, was the limited supply of residual patient material for further analysis. DNA was in short supply to both optimise and carry out molecular testing for *ompA* and *ompB* as is often recommended for *Rickettsia* speciation and phylogenetic analysis (Fournier et al., 2008, Fournier and Raoult, 2007, Fournier et al., 2003). *OmpA* and *ompB* PCR assays were optimised using diluted cell-line American Type Culture Controls (ATCC) as reactive controls, but when the patient DNA samples were tested using these optimised techniques, no amplicons could be detected. It is suspected that the *ompA* and *ompB* assays lacked the sensitivity to detect rickettsial DNA from the human samples and assays should ideally have been optimised using known 'natural infection' controls. This was unfortunately not possible given the limited amount of residual DNA. The recommended sample type for *Rickettsia* testing is eschar swab or eschar biopsy, as it reportedly has a higher concentration of the bacteria (Harrison et al., 2016, Althaus et al., 2010). Nevertheless, there exists support for using the faster-evolving *gltA* encoding gene as a target region for both diagnostic identification and phylogenetic analysis,

despite its reliability being limited to showing phylogenetic inter-relationships of rickettsiae undergoing early divergence from the common SFG ancestors (Fournier et al., 1998a, Fournier et al., 2003, Guillemi et al., 2015). In this study, it was found that the severe disease outcomes could be attributed to *R. conorii* based on the *gltA* region. It has been previously reported, that patients suspected of having CCHF/VHF due to severe or fatal outcomes, were found to be PCR positive for *R. conorii* (Frean et al., 2008, Kemp et al., 2014, Frean and Grayson, 2019). Unfortunately, the sequences obtained from this study could not be compared with other *gltA* sequences from South Africa, as primers were designed ‘in-house’ and amplified a different region on the gene than was published elsewhere. It is therefore recommended that future similar studies use well-cited and well-designed primers for which sequencing data is available to facilitate alignments and comparative analyses.

5.5 CONCLUSION

In conclusion, it is of paramount importance that clinicians become sensitized to the prospect that a usually benign and self-limiting disease in South Africa, may cause severe and fatal patient outcomes in some patients. In cases that are suspected of being CCHF/VHFs, TBF should also be investigated as a possible aetiology for the disease and the source of the exposure and the symptoms often overlap. Clinicians are encouraged to submit eschar swabs and/or eschar biopsies, where available, for PCR analysis in addition to the blood samples submitted, as the likelihood of detecting *Rickettsia*, if present, is improved. Antibiotic treatment should not be delayed pending laboratory confirmation and patients should receive treatment as early as possible (Frean et al., 2008).

CHAPTER 6 - BIOSURVEILLANCE OF RICKETTSIA IN TICKS COLLECTED AT SELECTED LOCATIONS IN SOUTH AFRICA

6.1 INTRODUCTION

Vector-borne zoonotic diseases (VBZD) are increasingly becoming recognised as a major public health threat worldwide, with reports of zoonoses accounting for approximately 2.5 billion cases and 2.7 million deaths globally, notwithstanding the vast economic burdens associated with these diseases (Saydam et al., 2021, Laukaitis and Macaluso, 2021). The public health burden of tick-borne rickettsioses is said to be significantly underestimated (Kim, 2022). Reports of over 100,000 cases of zoonotic infections are vector-borne, SFG rickettsioses caused by *Rickettsia* species considered among the most widespread emerging and re-emerging zoonotic infections transmitted by arthropod vectors (Katswara and Mukaratirwa, 2021). Of the arthropod vectors, ticks are thought to transmit the largest diversity of pathogenic micro-organisms, including protozoa, bacteria and viruses, more than any other arthropod vector group (Chitanga et al., 2014, Estrada-Pena and Jongejan, 1999, Estrada-Pena et al., 2013, Kisten et al., 2021, Pillay et al., 2022). In a recent study reviewing publications pertaining to VBZD, it was reported that hard ticks (family Ixodidae) are the vectors most commonly involved in the transmission of VBZD accounting for around 40% of documented emerging VBZD, most notably rickettsiae (Swei et al., 2020). The full impact of specifically TBD in sub-Saharan Africa, remains difficult to assess due to the insufficiency of available information on the prevalence, distribution and burden of neglected tropical diseases caused by TBDs (Chitanga et al., 2014, Katswara and Mukaratirwa, 2021). Deficient surveillance for ticks and TBDs has reportedly resulted in the under- and missed diagnoses of tick-borne infections and therefore the surveillance of ticks and their pathogens is essential for the effective investigation and management of TBDs (Ledger et al., 2021). There are at least 88 hard tick species found in Southern Africa, 11 of which belonging to the genus *Amblyomma* (Horak et al., 2018, Tarragona et al., 2021). Ticks primarily feed on animals, with humans being incidentally fed on in the absence of their preferred animal hosts. As a result TBPs

are incidentally transmitted to humans who are considered ‘dead-end’ hosts that do not contribute to their circulation and proliferation in nature (van Wyk et al., 2022, Kim, 2022).

In SA, there are four (4), tick-borne *Rickettsial* species said to be of medical importance, namely *R. africae* (Kelly et al., 1996), *R. aeschlimannii* (Beati et al., 1997), *R. conorii* (Brumpt, 1932) and *R. sibirica mongolitimonae* (Chitanga et al., 2014, Katswara and Mukaratirwa, 2021, Ledger et al., 2021). It is widely accepted that *R. africae*, the causative agent of ATBF, is primarily transmitted by the aggressive, hunting *Amblyomma* genus, specifically *Amblyomma hebraeum* in SA (Althaus et al., 2010, Kelly et al., 1992, Chitanga et al., 2014, Ledger et al., 2021, Ledger et al., 2022). *R. aeschlimanni* is thought to be transmitted by *Rhipicephalus appendiculatus* (Pretorius et al., 2004, Chitanga et al., 2014), and *R. conorii* is primarily transmitted by the brown dog tick, *Rh. sanguineus* (Parola et al., 2005a, Chitanga et al., 2014), but has also been reportedly isolated from *Haemaphysalis* ticks (Kelly et al., 1992, Chitanga et al., 2014). *Rickettsia sibirica mongolitimonae*, the causative agent of lymphangitis-associated rickettsiosis (LAR) has been associated with several tick species of the genus *Hyalomma* (Pretorius and Birtles, 2004a, Katswara and Mukaratirwa, 2021). Second only to malaria, rickettsioses are the leading cause of acute febrile illnesses among travelers to Southern Africa (Ledger et al., 2022, Lee et al., 2022, Tarragona et al., 2021, Qiu et al., 2021, Peter et al., 2021) and with increased eco- and wildlife tourism, ever-growing human populations, and continuously evolving land-use patterns and livestock husbandry activities, the probability of humans encountering *Rickettsia*-carrying ticks too increases (Tarragona et al., 2021, Silva-Ramos and Faccini-Martínez, 2021, Peter et al., 2021). Ticks and TBPs are however, not confined to rural settings with several tick species becoming well adapted to metropolitan environments (van Wyk et al., 2022). Furthermore, the expansion and invasion of ticks and their pathogens into new geographical areas, has been aided by climate change and urban encroachment leading to the establishment of new epicenters of TBDs (Kim, 2022). This adaptation is highlighted in publications reporting tick egg-laying in wall cracks and within companion animal bedding, causing tick incursions in human habitats (Dantas-Torres, 2008, van Wyk et al., 2022). A recent study conducted

by van Wyk et. al. (2022) in Potchefstroom, SA investigated the harborage of TBPs collected from ticks on stray dogs in the area and reported a 14.3% (7/49) prevalence of *Rickettsia* species in *Haemaphysalis elliptica* ticks and a 5.5% (3/55) prevalence in *Rh. sanguineus* ticks (van Wyk et al., 2022).

In this study, the objective was to determine the harborage of *Rickettsia* species in randomly-collected ticks collected in SA.

6.2 MATERIALS AND METHODS

6.2.1 STUDY SITES

The ticks were collected opportunistically from various locations and various sources including vegetation and animal livestock hosts on a privately-owned holiday farm in Limpopo Province and from privately-owned livestock farms in Northern Cape and Free State Provinces, respectively. In addition, a large collection of randomly collected ticks from various localities were donated for testing.

6.2.1.1 Lekkerbreek farm, Bela-Bela, Limpopo Province

Bela-Bela, named after the geothermic hot springs within it, is a town in the Waterberg District in Limpopo Province of SA, just off the N1 freeway between Pretoria and Polokwane (previously known as Pietersburg). The greater Bela-Bela is a 4,000 km² area scattered with nature reserves, farmlands and its infamous hot springs, and is classified as both urban and rural with the municipality sharing borders with Gauteng, Mpumalanga and North West Provinces. The prevalent climate in Bela-Bela is a local steppe climate with little rainfall seen throughout the year (rainy season is October to March) the rainfall is 636 mm and the average annual temperature is 19.6°C³ and has an average elevation of approximately 3,737 feet above sea level.

³ <https://en.climate-data.org/africa/south-africa/limpopo/bela-bela-26798/>

The sampling area is described as a rural ‘bushveld’ savannah setting with dense vegetation including acacia trees, shrubs and long grass, supporting a sizable population of wild herbivorous game. The area is used for recreational ‘eco-tourism’ where there are several cottages strewn along the southern bank of a small dam used by residents for fishing. There is very little development in this area, as shown in the satellite picture (Figure 6.1) and photographs of the path along which ticks were collected (Figure 6.2). Sampling was convenience-based and dependent on the availability of ticks at the particular location. Ticks were picked directly from the foliage along a dirt-path towards the dam. The area is known to be endemic to *Rickettsia* as there have been reported cases of TBF in returning travelers from the area (personal communication). The prevalent tick species in the area is unknown, but sampling has provided an abundance of *Rhipicephalus* species ticks. A study conducted by Schroder and Reilly (2013) which looked at tick species diversity in the Waterberg area, Limpopo Province, South Africa reported that the most abundant tick species collected in the area was *Rhipicephalus (Boophilus) decoloratus*, and that a total of eight (8) tick species were collected from the area, i.e. *Amblyomma hebraeum*, *Rhipicephalus (Boophilus) decoloratus*, *Haemaphysalis elliptica*, *Hyalomma rufipes*, *Rhipicephalus appendiculatus*, *Rhipicephalus evertsi evertsi*, *Rhipicephalus zambeziensis* and *Rhipicephalus* species (Schroder and Reilly, 2013).



Figure 6.1 (A) Map and (B) Satellite image of the area sample collection was carried out. GPS co-ordinates -24.828762, 28.214527. Map and satellite picture captured via Google Earth.

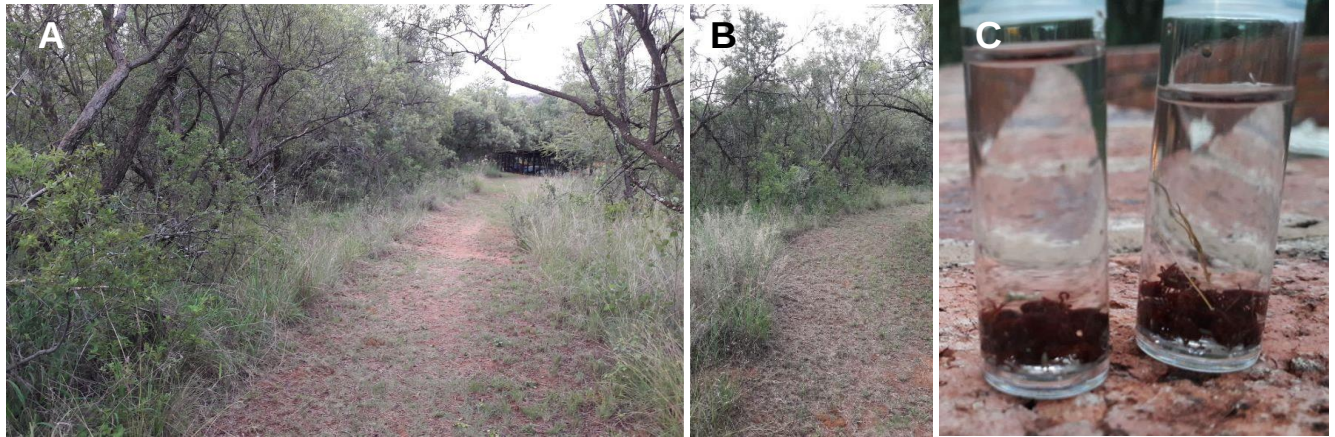


Figure 6.2 Photographs taken during tick sample collection illustrating the vegetation along the pathway to the dam, from which the ticks were collected (A & B). The ticks collected were placed directly into vials containing 70 % ethanol (C) and brought to the laboratory for processing. Photographs courtesy: Dr. J Weyer.

6.2.1.2 Free State Province

Ticks were collected from several privately-owned farms in the southern-western Free State Bloemfontein (29.0852°S, 26.1596°E) and eastern parts of the Northern Cape Province, Mokala National Park (29.1659°S, 24.3197°E) in an approximately ~40,000 km² (200 km by 200 km), at area 994–1794 m above sea level. The area is considered rural temperate grassland, classified as highveld grasslands, in a more semi-arid part of South Africa, especially on the inland plateau and is considered a cold semi-arid steppe climate. The mean annual rainfall ranges between 300–600 mm (Msimang et al., 2021) (Figure 6.3). The region is known to be endemic to four (4) major tick vector species, namely *A. hebraeum*, *R. appendiculatus*, *R. decoloratus* and *Ixodes rubicundus* as well as the non-endemic, but well established species *R. microplus* (Horak et al., 2015), but *Hyalomma* species (i.e. *Hyalomma marginatum rufipes* and *Hyalomma truncatum*) have also been reported to occur in the region (Dreyer et al., 1998). Although *Hyalomma* species ticks are ubiquitous throughout SA, steppe and savannah semi-arid regions reveal concentrated *Hyalomma* populations (Msimang et al., 2021).

Convenience-based sampling was conducted, depending on the availability of the ticks. The ticks were collected as part of the 2015-2017 Eco-Health Alliance project entitled:

‘Seroprevalence and Infection Prevalence Study in Humans and Vectors in an Endemic area of Transmission of Crimean-Congo haemorrhagic fever virus, South Africa, 2015 – 2019.’ The arthropods were collected directly from animal hosts (mostly cattle) (with the highest concentration of ticks at the perineum area, around the hooves and in the tail brush) or from the ground whilst the ticks were seeking a new host. *Hyalomma* species were specifically targeted for that study, therefore traditional flagging or dragging techniques were less effective and unsuitable for hunting *Hyalomma* ticks. The ticks were collected from the animals using thumb forceps and transferred to a 15 mL pierced vial. The ticks were kept alive in a field refrigerator at 4°C and transported to the NICD, CEZPD/SVPL laboratory for morphological species identification and processing. Ticks were stored at 4°C until processing.

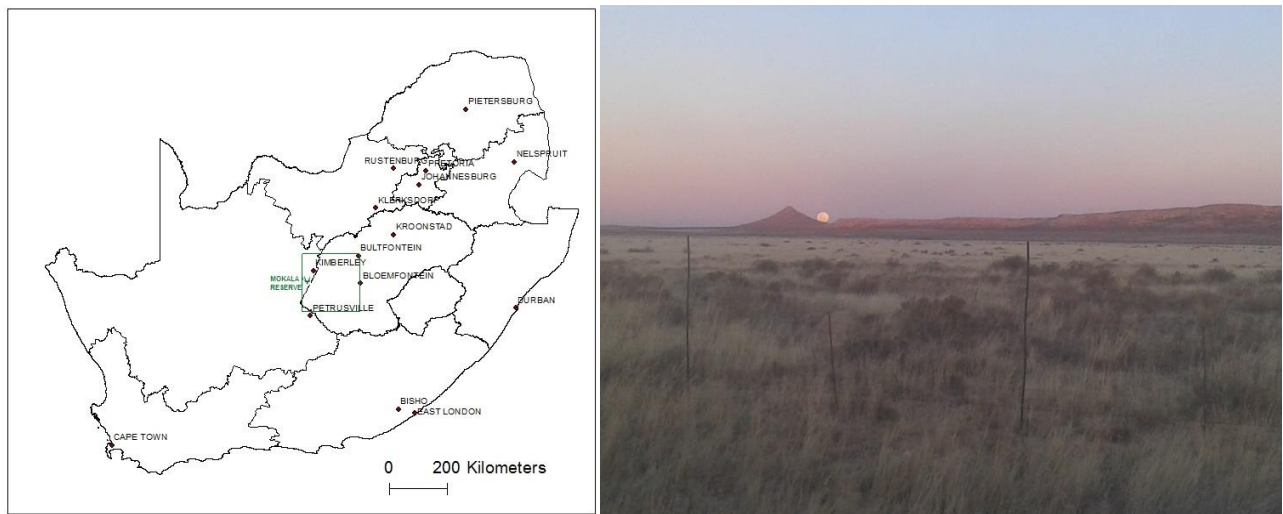


Figure 6.3 Left, map illustrating general location of sample site south-western Free State Bloemfontein (29.0852°S, 26.1596°E) and eastern parts of the Northern Cape Province, Mokala National Park (29.1659°S, 24.3197°E); Right, photograph illustrating the semi-arid grassland terrain of the sample area. Photographs courtesy of Dr. V Msimang.



Figure 6.4 Tick sample collection at tick feeding sites on livestock hosts and sample preparation prior to transport to the NICD. Photographs courtesy of Dr. V Msimang.

6.2.1.3 Donated Ticks

A historical collection of ticks of various species, collected from various areas around South Africa were donated by an acarologist at the Agricultural Research Council – Onderstepoort Veterinary Institute (ARC-OVI) from his private collection for *Rickettsia* testing. Ticks were collected from nine locations in three provinces (i.e. Onderstepoort farm in Gauteng Province, Hluhluwe Nature Reserve and St Lucia in KwaZulu-Natal Province and Bredasdorp, Stellenbosch, Franschoek, Houtbay, Noordhoek areas in Western Cape Province). Unfortunately, information regarding specific collection locations, sources (i.e. vegetation or from an animal host), date of collection and other factors were not available for all specimens. The specimens were however separated according to species and stored in 70% ethanol at 4°C from collection until processing. Various tick species were collected including *H. rufipes*, *H. truncatum*, *Rh. evertsi*, *Rh. appendiculatus*, *Rh. decoloratus*, *Rh. evertsi evertsi*, *Rh. gertrudae*, *Rh. maculatus*, *Rh. microplus* and *Rh. simus*.

6.2.2 RESEARCH PERMITS AND ETHICS

Animal ethical clearance was sought from the University of Pretoria's Animal Ethics Committee for the sampling and testing of ticks; however, it was considered outside the scope of the committee's mandate since the focus was on the arthropod vectors and not

on vertebrates. Specimen collection from cattle was however approved by the Animal Ethics Committee, University of Pretoria, South Africa (protocol no. v020-17 and t005-16). Section 20 approval (protocol no. 12/11/1/1/13) was obtained from the Department of Agriculture, Land Reform and Rural Development (DALRRD), project reference 131/11 (exp. 12/2020) (see supplementary material - Appendix 6.1). Consent to collect specimens from cattle was obtained from all farm owners in the Free State and Northern Cape farms and no special permission was required to collect from the Lekkerbreek Farm, as the ticks were collected from the foliage by a co-owner of the farm.

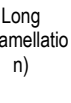
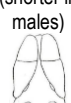
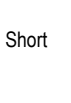
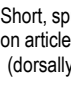
6.2.3 TICK COLLECTIONS

All ticks were removed from cold storage (4°C) and allowed to reach ambient room temperature before attempting morphological identification as ticks are fragile when cold and legs were required to aid identification. The Limpopo-caught ticks were removed from the 70 % ethanol in which they were stored and allowed to air dry on a piece of filter paper before being rinsed in phosphate-buffered saline (PBS) (pH~7.2) and again allowed to air-dry on a separate piece of filter paper that was then placed onto the base-lit stereo-microscope for positioning and identification. The ticks collected from the Free State were systematically washed using deionised water and allowed to air-dry prior to morphological identification using a stereo microscope.

Adult tick gender is distinguishable based on various morphological features. The most obvious difference between males and females is the single, large hardened dorsal surface of a male tick, called the conscutum; versus the female's bi-segmented dorsal surface, hardened, rigid scutum and softer, expanding alloscutum. The ticks were initially separated into males and females based on this feature. Once the genders were separated, the key (Table 6.1) was used to determine the genus. Specific features assessed to determine the tick genus included; length of the palps, enameling patterns, presence or absence of eyes, presence or absence of eleven festoons, anal groove

shape and the appearance of legs. The adanal plates and the presence or absence of accessory adanal plates aided in genus identification for the male ticks.

Table 6.1. South African key of Ixodid genera (summarized from Walker, 2003).

IXODID GENERA OF SOUTH AFRICA											
	Amblyomma	Amblyomma (Aponomma)	Cosmionomma	Dermacentor	Ixodes	Haemaphysalis	Hyalomma	Margaropus	Rhipicephalus	Rhipicephalus (Boophilus)	Rhipicephalus
Palps	Long 	Long 	Long (enamellation) 	Medium 	Long, thin (shorter in males) 	Short, very triangular, article 2 extended laterally 	Long 	Short 	Medium 	Very short 	Short, spur on article 1 (dorsally) 
Enameled pattern	Yes	Variable	Yes	Yes	No	No	No	No	Variable	No	No
Eyes	Flat to Hemispherical	Absent	Hemispherical	Present	Absent	Absent	Orbited	Present	Present	Present	Present
Number of festoons	11	11	11	11	Not present	11	<11	Not present	11	Not present	11
Shape of anal groove											
Adanal plates (males only)	No adanal plates	No adanal plates	Single set of anals	No adanal plates	Several flat plates	No adanal plates	3 sets (anals, accessory and subanal)	Anals and subanals	Anals ± subanals	Anals and subanals	No adanal plates
Legs	Annulated	Uniform	Enamelled	Annulated	Slender and uniform	Slender and uniform	Annulated with a dorsal strip	Yellow-brown, annulated, strongly beaded	Uniform or annulated	Slender, yellow, slightly beaded	Uniform
Other unique features (females)	 <i>Amblyomma</i>		Alloscutum: soft patches 	Alloscutum: setose patches 	 <i>Ixodes</i>	 <i>Haemaphysalis</i>			 <i>Rhipicephalus</i>	 <i>Boophilus</i>	
Other unique features (males)	Dorsal scutes and ventral platelets			Enlarged coxa 4	Marginal groove (entire)			Enlarged leg 4, process: dorsal and subterminal	Caudal process ±	Caudal process present	Enlarged coxa 4, long spurs (adanal)

6.2.4 DNA EXTRACTIONS

Ticks were removed from the refrigerator (4°C) and allowed to reach ambient room temperature before being processed. Due to the plethora of TBD harboured by ticks, possibly including CCHF virus, all ticks were processed in a class 2 A2 biological safety cabinet (BSC) with appropriate disposable personal protective equipment (PPE) including gloves and disposable overcoat.

The ticks were then individually placed into a 2 mL screw-cap tubes containing a 5 mm diameter stainless steel tissue lysis bead (Qiagen, Hilden, Germany), and 500 µL of Eagle's minimum essential medium with triple antibiotic solution concentration (penicillin 10,000 IU/ mL, streptomycin 10 mg/mL and amphotericin B 25µg/mL cell culture medium). The ticks were then subjected to manual tissue lysis using a Qiagen Tissue Lyser (Qiagen, Hilden, Germany). Ticks were homogenised at 30 Hz for 8 min due to the hard/leathery tick exoskeletons, the tissue lyser had to be run at top speed for longer than is usually recommended to ensure complete manual lysis and homogenisation was achieved. The homogenates were then centrifuged for 2 min at 6,000 *g* in a benchtop microcentrifuge to allow the tick debris to form a pellet on the bottom of the tube. The lysed samples were then frozen at -20°C until testing.

DNA was extracted from each individual tick homogenate using the QiAmp Viral RNA kit (Qiagen, Hilden, Germany) on the QIAcube automated extractor (Qiagen, Hilden, Germany). A-hundred-and-forty microliters (140 µL) of the tick homogenates were transferred into a separate 2 mL screw-cap tubes containing 560 µL of prepared Buffer AVL. The mixtures were then thoroughly homogenised by pulse-vortex for 15 s, followed by a 10 min incubation at ambient room temperature. The mixture was then briefly centrifuged to remove droplets from the inside of the lid, and loaded onto the QiaCube automated extractor to a maximum of 12 per run for the remainder of the extraction steps as per manufacturer standard protocol. The final DNA/RNA elution volume was 60 µL of buffer AVE.

The DNA extracted from the individual ticks of the same species (morphologically identified) were then pooled (five (5) ticks per pool) by transferring 10 µL aliquots of tick DNA into a separate ‘pooling’ microtube, to be used as the template DNA pools for screening PCR. If a pool tested positive, the unpooled, component tick DNA samples were subsequently individually tested by PCR to determine which of the component ticks were harbouring *Rickettsia* species.

6.2.5 PCR ASSAYS

6.2.5.1 *Rickettsia* PCRs

All PCRs were set up using the Expand™ High Fidelity PCR System (Roche Diagnostics GmbH, Mannheim, Germany) in accordance with manufacturer protocols and in-house optimisation using the Taguchi method (Cobb and Clarkson, 1994). Primers designed in-house were used to target the *gltA* region, and published primers were selected to target the outer-membrane proteins rOmpA and rOmpB (*ompA* and *ompB*) genes of SFG *Rickettsia* (see Table 6.2). Each reaction contained 10 µL of Expand High Fidelity Buffer (10 ×) with 15 mM MgCl₂, 200 µM of each dNTP, 0.3 µM of a particular primer pair (either *gltA*, *ompA* or *ompB*), 2.6 U Expand High Fidelity Enzyme mix, 10 µL of extracted DNA and nuclease-free water to a final total reaction volume of 50 µL. Reactions were amplified on a Veriti® Thermal cycler (Applied Biosystems Thermo Fisher Scientific) under the following conditions: initial denaturation step of 94°C for 2 min followed by 40 cycles of denaturation at 94°C for 20 sec; annealing at 47°C for 30 sec, extension at 68°C for 30-60 sec; and a final extension at 68°C for 5 min and hold at 4°C (infinite). PCR products were electrophoresed at 80 V for 45 – 60 min on 1.0% (w/v) TBE-agarose gels, stained with Gel Red (Biotium, Inc., USA) and size fractionated using a 100 bp molecular weight marker (mwm) (Bioline, UK).

Tick DNA pools were initially subjected to a *gltA* screening round in order to identify whether there were any *Rickettsia*-positive ticks in the pool whilst reducing wastage of reagents for testing negative ticks. If a tick pool tested *Rickettsia*-positive, a set of five (5)

gltA reactions were set up to test each of the ticks that made up the *Rickettsia*-positive pool, in order to identify which of the ticks were harbouring the *Rickettsia*. Only *gltA*-positive ticks were then subjected to *ompA* and *ompB* PCRs. All positive amplicons (*gltA*, *ompA* and *ompB*) were ‘cleaned up’ and sequenced as described in Sections 6.2.6 and 6.2.7 respectively. Figure 6.5 shows the expected PCR amplicon sizes for three (3) targeted genes. *Note: the figure is a visual representation of a positive control and is for demonstrative purposes only.* .

Table 6.2 PCR primers used for the identification and characterization of *Rickettsia* species targeting the citrate synthase gene (*gltA*), and outer membrane proteins A and B (*OmpA* and *OmpB*)

Gene	Primer name	Primer sequence (5' → 3')	Expected product size (bp)	Reference
<i>gltA</i>	Rick F	GACGGTATACCCATACGTTTATAG	333	Designed in-house
	Rick R	TAATGAAGCGGTAATAAATATG		
	RpCS.877p	GGGGGCTGCTCACGGCGG	382	(Oteo et al., 2006)
	RpCS.1258n	ATTGCAAAAAGTACAGTGAACA		
<i>OmpA</i>	Rr190.70pa	ATGGCGAATATTTCTCCAAAA	629 – 632	(Regnery et al., 1991, Oteo et al., 2006, Roux et al., 1996)
	RR190.701R	GTTCCGTTAATGGCAGCATCT		
<i>OmpB</i>	120-2788	AAACAATAATCAAGGTACTGT	812 – 815	(Roux and Raoult, 2000)
	120-3599	TACTTCCGGTTACAGCAAAGT		

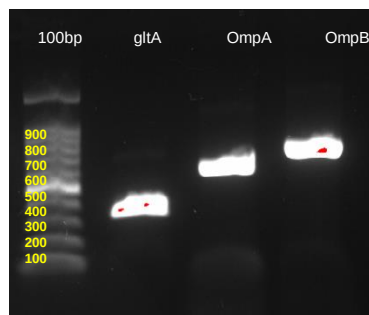


Figure 6.5. Controls: Citrate synthase (*gltA*), Outer membrane proteins A (*OmpA*) and B (*OmpB*) positive rickettsial PCR products stained with Gel Red and electrophoresed through a 1.0% (w/v) TBE-agarose gel (80V for 45 min). Sizes: *gltA*:~382bp, *OmpA*:~629bp, *OmpB*:~815bp

6.2.5.2 Molecular identification of ticks

Ticks were morphologically identified for this study, however, these identifications were confirmed using PCR reactions targeting appropriate regions in the tick genomes depending on genus, i.e. *Rhipicephalus* or *Hyalomma*. Published primers were selected as per Table 6.3. Two (2) regions were targeted for the *Rhipicephalus* identifications, i.e. the 18S ribosomal DNA (18S rRNA) and internal transcribed spacer 2 (ITS-2) (Abdullah et al., 2016, Latrofa et al., 2013), and one (1) region for the *Hyalomma* identifications, i.e. mitochondrial DNA (mtDNA) cytochrome C oxidase subunit I (COI) (Keve et al., 2023, van Wyk et al., 2022, Uiterwijk et al., 2021, Licari et al., 2017, Lv et al., 2014, Hebert et al., 2003).

Table 6.3 PCR primers used for the identification of *Rhipicephalus* species targeting the 18S ribosomal DNA (18S rRNA) and internal transcribed spacer 2 (ITS-2), and for the identification of *Hyalomma* species targeting the mtDNA cytochrome C oxidase subunit I (COI).

Genus	Gene	Primer Name	Primer sequence (5' → 3')	Expected product size (bp)	Annealing temperature (°C)	Reference
Rhipicephalus ¹	18S rRNA	18S-F	CATTAAATCAGTTATGGTTCC	810	45	(Abdullah et al., 2016, Latrofa et al., 2013)
		18S-R	CGCCGCAATACGAATGC			
	ITS2	ITS2-F	ACATTGCGGCCTTGGGTCTT	1053 – 1223	54	
		ITS2-R	TCGCCTGATCTGAGGTCGAC			
		ITS2 F Int	GAGCGAAAGCCGGCCAGC	Internal sequencing primers		Designed in-house (This study)
		ITS2 R Int	GCGTGTTGAGCGCCCGG			
Hyalomma ¹	COI	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	658 - 710	47	(Hebert et al., 2003)
		LCO1490	GGTCAACAAATCATAAAGATATTGG			

¹ Alignments demonstrating primer binding positions are provided in Supplementary material (Appendices)

All PCRs were set up using the GoTaq® DNA Polymerase (Promega, WI, USA) in accordance with manufacturer protocols and in-house optimisation using the Taguchi method (Cobb and Clarkson, 1994).

6.2.5.2.1 *Rhipicephalus* I8S PCR

Each reaction contained 10 µL of Colorless GoTaq® Reaction Buffer (5x), 2 mM MgCl₂, 200 µM of each dNTP, 0.6 µM of each (18S) primer as per Table 6.3 (also see supplementary materials for binding positions of primers) , 1.5 U GoTaq® DNA Polymerase, 1 µL of extracted tick DNA and 27.8 µL nuclease-free water to a final reaction volume of 50 µL. Reactions were amplified on a Veriti® Thermal cycler (Applied Biosystems Thermo Fisher Scientific) under the following conditions: initial denaturation step of 95°C for 2 min followed by 35 cycles of denaturation at 95°C for 30 sec; annealing temperature at 45°C (as per Table 6.3) for 30 sec, extension at 72°C for 30 sec; and a final extension at 72°C for 6 min and hold at 4°C until the products were removed for electrophoresis. Five microliters (5 µL) of each PCR product was mixed with a microliter (1 µL) of blue-orange loading dye (x6) (Promega, WI, USA), and loaded into 5 mm wells on a 1 – 1.2% (w/v) TBE-agarose (Promega, USA) gel, stained with 1:20 000 final dilution Gelred (Biotium, Inc., USA) and size fractionated using the 100 bp mwm (Promega, USA). The gels were electrophoresed at 80 V for 60 min and visualized on a ChemiDoc™ XRS+ with Image Lab™ Software (Biorad, CA, USA) molecular imager.

6.2.5.2.2 *Rhipicephalus* ITS2 PCR

The ITS2 PCR reactions contained 10 µL of Colorless GoTaq® Reaction Buffer (5x), 2.5 mM MgCl₂, 200 µM of each dNTP, 0.2 µM of each ITS2 outer primer (i.e. ITS2-F and ITS2-R) as per Table 6.3 (also see supplementary materials for binding positions of primers) , 1.5 U GoTaq® DNA Polymerase, 1 µL of extracted tick DNA and 30.8 µL nuclease-free water to a final reaction volume of 50 µL. Reactions were amplified as described in section 6.2.5.2.1 on a Veriti® Thermal cycler (Applied Biosystems Thermo Fisher Scientific) with the only difference being the annealing temperature that was set at 54°C (as per Table 6.3) for 30 sec. PCR products were then electrophoresed and visualized as described in 6.2.5.2.1.

Due to the large product size amplified by the ITS2 primers, internal sequencing primers had to be designed to assure a greater product size is available for species identification. These internal primers are provided in Table 6.3 and binding positions are demonstrated in the supplementary materials section.

6.2.5.2.3 *Hyalomma* COI PCR

The COI PCR reactions contained 5 µL of Colorless GoTaq® Reaction Buffer (5x), 3 mM MgCl₂, 200 µM of each dNTP, 0.4 µM of each COI outer primer (i.e. HCO2198 and LCO1490) as per Table 6.3, 1.5 U GoTaq® DNA Polymerase, 1 µL of extracted tick DNA and 12.8 µl nuclease-free water to a final reaction volume of 25 µL. Reactions were amplified as described in section 6.2.5.2.1 on a Veriti® Thermal cycler (Applied Biosystems Thermo Fisher Scientific) with the annealing temperature that was set at 47°C (as per Table 6.3) for 30 sec. PCR products were then electrophoresed and visualized as described in 6.2.5.2.1.

6.2.6 PCR AMPLICON PURIFICATION AND SANGER SEQUENCING

All *Rickettsia* (*gltA*) PCR-positive samples were subjected to various subsequent dideoxynucleotide cycle sanger sequencing for identification purposes as described for section 6.2.5. Amplicons of appropriate/expected size were purified using the Wizard® SV Gel and PCR Clean-up System (Promega, Madison, WI, USA) as per manufacturer protocol. The methodology is described in Chapter 5, Section 5.2.7. The eluted, purified products were then subjected to sanger sequencing. The purified amplicon concentrations (in ng/µL) were measured for purity (i.e. 260/280 ratio >1.8) and quality (i.e. 260/230 ratio in the range of 2.0-2.2) using the Thermo Scientific™ NanoDrop 2000 and 2000c.

Direct sequencing reactions were set up using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Scientific, Carlsbad, CA, USA). Briefly, 2 µL of BigDye™ Ready Reaction Mix, 1 µl BigDye™ Terminator v1.1 & v3.1 5X Sequencing Buffer, and 10 µM of either forward or reverse primer was added to

approximately 100 ng of template amplicons and made up to a 10 µL total reaction volume with deionized (RNase/DNase-free) water. Reactions were then placed onto a thermo cycler and subjected to sequencing cycle conditions as follows: 96°C for 60 sec; followed by 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min with a 1°C/sec ramp rate. The reactions were then held at 4°C until purification of sequencing reactions.

Purification of sequencing reactions was performed in one (1) of two (2) ways; i.e. either using the Centri-Sep™ columns or using the BigDye XTerminator™ method. Both methods are recommended for sequencing and selection of method was dependent on the availability of kits in the lab during processing with no preference or notable difference in the final outcomes. Centri-Sep™ columns (Princeton Separations, Adelphia, NJ, USA) were used as per manufacturer protocols to clean-up the sequencing reactions (predominantly *gltA*, *OmpA* and *OmpB* sequencing reactions), which were then transferred to 0.2 mL microtubes, and allowed to evaporate at 95°C for 10-25 min (depending on volume) on a thermocycler. Ten microliters (10 µL) of Hi-Di™ Formamide (Applied Biosystems, Thermo Fisher Scientific, USA) was then applied to the desiccated sequencing reactions, which were then returned to the thermocycler for 3 min at 95°C to allow for denaturation to occur. The Formamide mixtures were then transferred to a MicroAmp Optical plate (Applied Biosystems, Thermo Fisher Scientific, USA) to be loaded onto an ABI 3500 DNA Sequencer (50 cm capillaries) with POP-7™ separation matrix (Applied Biosystems, Thermo Fisher Scientific, USA) for sanger sequencing. The BigDye XTerminator™ method did not require sequencing reactions to be purified, desiccated and resuspended in preparation for sequencing. For each 10 µL sequencing reaction; 10 µL BigDye XTerminator™ bead solution was homogenised with 45 µL of SAM solution in a 'SAM/BigDye XTerminator™ bead working solution'. The SAM/BigDye XTerminator™ bead working solution was vortexed throughout pipetting to ensure the beads remained suspended in the mixture whilst being applied to the MicroAmp™ Optical 96-well reaction plate. The 10 µL sequencing reactions were then applied directly to the bead working solution-containing reaction plate wells and sealed using a MicroAmp™ Clear Adhesive Film. The 96-well plate was then vortexed at 1800 rpm for 20 min, and

briefly centrifuged at 1000 x *g* for 2 min before being loaded onto an ABI 3500 DNA Sequencer (Applied Biosystems) as described above.

6.2.7 SEQUENCE DATA ANALYSIS

Sequence data was analysed using BioEdit version 7.2.5 (BioEdit Sequence Alignment Editor© 1997-2013 Tom Hall). Both strands were sequenced, pairwise aligned and a consensus sequence was constructed for each amplified region; i.e. *gltA*, *rOmpA*, and *rOmpB* for *Rickettsia* identification (Regnery et al., 1991, Oteo et al., 2006, Roux et al., 1996); 18S rRNA and ITS-2 for *Rhipicephalus* identification (Abdullah et al., 2016, Latrofa et al., 2013); and COI for *Hyalomma* identifications (Keve et al., 2023, van Wyk et al., 2022, Uiterwijk et al., 2021, Licari et al., 2017, Lv et al., 2014, Hebert et al., 2003) using the BioEdit sequence alignment editor (Hall, 1999).

Consensus sequences were compared with those on GenBank using the NCBI BLAST 2.0 tool (National Centre for Biotechnology Information, Bethesda, MA, U.S.A.; <http://www.ncbi.nlm.nih.gov/blast/>) for homology searching. A multiple-sequence ClustalW alignment was performed (Thompson et al., 1994, Fournier et al., 2003), aligning the consensus sequences with similar sequences (as tabulated in Tables 6.4 – 6.5) using the MEGA (version 11.0.13) software. Sequences were edited at the 5' and 3' ends so that sequence lengths matched that of the longest sequence common to all compared species (Fournier et al., 2003).

Table 6.4 Genbank accession numbers of *Rickettsia* gene sequences used for comparative purposes

Validated species	Species strain	GenBank accession no.		
		<i>GltA</i>	<i>OmpA</i>	<i>OmpB</i>
<i>R. aeschlimannii</i>	MC16T	U59722	U43800	AF123705
<i>R. africae</i>	ESF-5	U59733	U43790	AF123706
<i>R. akari</i>	MK (Kaplan), ATCC VR-148T	U59717	N/A	AF123707
<i>R. australis</i>	Phillips	U59718	AF149108	AF123709
<i>R. bellii</i>	369L42-1	U59716	N/A	N/A
<i>R. canadensis</i>	2678	U59713	N/A	N/A
<i>R. conorii</i>	Seven (Malish), ATCC VR-613T	U59730	U43806	AF123721
<i>R. conorii</i>	Indian tick typhus <i>rickettsia</i> , ATCC VR-597	U59730	U43794	AF123726
<i>R. felis</i>	URRWXCal2, ATCC VR-1525	AF210692	AF210694	AF210695
<i>R. helvetica</i>	C9P9	U59723	N/A	AF123725
<i>R. honei</i>	TT-118, ATCC VR-599T	U59726	U43809	AF123724
<i>R. japonica</i>	YM	U59724	U43795	AF123713
<i>R. massiliae</i>	MtutT	U59719	U43799	AF123714
<i>R. montanensis</i>	M/5–6	U74756	U43801	AF123716
<i>R. parkeri</i>	Maculatum 20	U59732	U43802	AF123717
<i>R. prowazekii</i>	Breinl, ATCC VR-142T	M17149	N/A	AF123718
<i>R. rickettsii</i>	R (Bitterroot), ATCC VR-891T	U59729	U43804	X16353
<i>R. rhipicephali</i>	3–7–6	U59721	U43803	AF123719
<i>R. sibirica</i>	246, ATCC VR-151T	U59734	U43807	AF123722
<i>R. slovaca</i>	13-B	U59725	U43808	AF123723
<i>R. typhi</i>	Wilmington, ATCC VR-144T	U59714	N/A	L04661

N/A - not available

Table 6.5 Genbank accession numbers for tick identification sequences

Validated species	Species strain	GenBank accession no.
<i>Rhipicephalus</i>		18S rRNA and ITS2 genes
<i>Rh. appendiculatus</i>	clone 6_8584_532	KY457501.1
<i>Rh. appendiculatus</i>	clone 14_8656_820	KY457500.1
<i>Rh. evertsi</i>	clone 2_9216_449	KY457503.1
<i>Rh. evertsi</i>	clone 2_8998_58	KY457502.1
<i>Rh. maculatus</i>	clone 50_9923_1162	KY457505.1
<i>Rh. maculatus</i>	clone 38_10044_457	KY457504.1
<i>Rh. microplus</i>	clone 60_8908_763	KY457506.1
<i>Rh. simus</i>	clone 7_9296_958	KY457508.1
<i>Rh. zambeziensis</i>	clone 221_9750_843	KY457509.1
<i>Rh. zambeziensis</i>	clone 307_9750_71	KY457507.1
<i>Hyalomma</i>		COI gene
<i>H. rufipes</i>	isolate Hrufi7	KU130627.1
<i>H. rufipes</i>	isolate HR80	JX049312.1
<i>H. rufipes</i>	isolate HR74	JX049308.1
<i>H. rufipes</i>	isolate Hy4_LE_QUT_2019_Hy543	MN453668.1
<i>H. rufipes</i>		AF132823.1
<i>H. rufipes</i>	isolate HR214	JX049329.1
<i>H. rufipes</i>	isolate Hr1_SA_FS_2018_Hr351	MK551197.1
<i>H. rufipes</i>	isolate HR6	JX049321.1
<i>H. rufipes</i>	HR56	JX049300.1
<i>H. turanicum</i>	isolate Hytu3	KT989638.1
<i>H. turanicum</i>	isolate Hytu2	KT989637.1
<i>H. turanicum</i>	isolate Hytu1	KT989636.1
<i>H. truncatum</i>	voucher CB1	KT999398.1
<i>H. truncatum</i>	voucher GH1	KT999413.1
<i>H. truncatum</i>	voucher FS1	KT999492.1
<i>H. truncatum</i>	voucher UP2	KT999475.1
<i>H. truncatum</i>	voucher SX1	KT999519.1

6.3 RESULTS

6.3.1 Morphological tick identifications

6.3.1.1 *Morphological identification of ticks collected from Limpopo Province.*

Morphological tick identification was aided by consultation with an acarologist at the ARC-OVI. The ticks collected from Limpopo Province were identified as *Rh. appendiculatus*, a species known to occur in Bela-Bela, Limpopo province. General features of *Rh. appendiculatus* include; a brown to darker-brown colouration without enamellation, flattened eyes, and small to medium sized punctations with smoother lateral areas.



Figure 6.6 Adult *Rhipicephalus appendiculatus* ticks (A) Female; (B) Male.

6.3.1.2 *Morphological identification of ticks collected from Free State*

Ticks collected from the Free State Province were morphologically identified by the principal investigator for the primary project for which the ticks were collected in consultation with a senior entomologist at the NICD. The ticks were identified using local keys and microscopic observation, and were determined to be *H. truncatum* and *H. marginatum rufipes*.

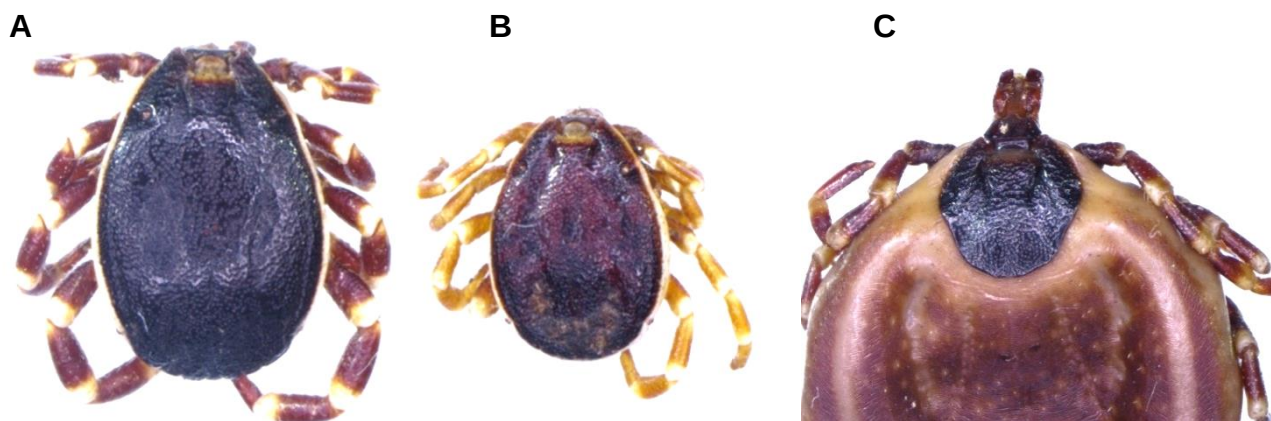


Figure 6.7 *Hyalomma marginatum rufipes*; (A and B) male; (C) female. Photographs courtesy of Mr. A Kemp

6.3.1.3 Morphological identification of donated ticks

All donated ticks were sorted and identified by the collector acarologist at the ARC-OVI using appropriate, area-specific keys and guides.

6.3.2 PCR screening of ticks for presence of *Rickettsia* using the citrate synthase gene (*gltA*)

A total of 374 *Rh. appendiculatus* ticks (320 females and 52 males) (Table 6.6) collected from the vegetation in Bela-Bela, Limpopo Province (as described in Section 6.2.1.1) were extracted and tested in 75 pools by *gltA* PCR for the presence of *Rickettsia* species. Two (2) of the 374 ticks provided low positives that could not be sequenced. The positives were repeated and subjected to semi-nested PCRs targeting the *gltA* gene, but did not test positive in subsequent attempts possibly due to template degradation, or presence of inhibitors in the template DNA. Due to the small size of the ticks, the entire tick was used up in the extraction process and extractions could therefore not be repeated.

A sub-sample of 273 ticks collected from the Free Province as part of the Eco-Health Alliance project described in section 6.2.1.2 were screened for *Rickettsia* as described previously. The majority of the ticks tested were *Hyalomma* species, specifically 138 *H. rufipes* (19 females and 119 males) and 130 *H. truncatum* (24 females and 106

males) (Table 6.6). The *H. rufipes* were found to have a 16% (22/138) positivity rate and none of the *H. truncatum* (0/130) were found to be carrying rickettsias. Of the positives detected, additional PCRs and sanger sequencing was conducted for samples of sufficient volume (i.e. > 50 µL template tick DNA), targeting the *gltA*, *rOmpA* and *rOmpB* genes to determine the *Rickettsia* species detected.

A total of 279 donated ticks (i.e. ten (10) species including *H. rufipes*, *H. truncatum*, *Rh. evertsi*, *Rh. appendiculatus*, *Rh. decoloratus*, *Rh. evertsi evertsi*, *Rh. gertrudae*, *Rh. maculatus*, *Rh. microplus* and *Rh. simus*) were extracted and screened for presence of *Rickettsia* as described previously. Five (5) male *Rh. simus* ticks collected from cattle in Gauteng Province (Table 6.6) were found to be *Rickettsia* positive by *gltA* screening. The positive specimens were subjected to *gltA*, *rOmpA* and *rOmpB* PCRs, followed by sanger sequencing to determine the *Rickettsia* species detected.

Table 6.6 Ticks screened for presence of *Rickettsia* using the *gltA* gene

	Location of collection	Tick species	Total tested	Gender		<i>gltA</i> PCR	
				F	M	NEG	POS
*	Limpopo province	<i>Rh. appendiculatus</i>	374	320	54	372	2
ECO-HEALTH	Free State Province	<i>H. rufipes</i>	138	19	119	116	22
		<i>H. truncatum</i>	130	24	106	130	0
		<i>Rh. evertsi evertsi</i>	1	0	1	1	0
		<i>Rh. appendiculatus</i>	4	0	4	4	0
DONATED TICKS	Gauteng Province	<i>H. rufipes</i>	2	2	0	2	0
		<i>H. truncatum</i>	3	2	1	3	0
		<i>Rh. appendiculatus</i>	15	5	10	15	0
		<i>Rh. decoloratus</i>	5	4	1	5	0
		<i>Rh. evertsi evertsi</i>	47	19	28	47	0
		<i>Rh. microplus</i>	22	14	8	22	0
		<i>Rh. simus</i>	12	0	12	7	5
	KwaZulu-Natal	<i>Rh. maculatus</i>	39	23	16	39	0
	Western Cape Province	<i>H. truncatum</i>	3	2	1	3	0
		<i>Rh. evertsi</i>	80	38	42	80	0
		<i>Rh. decoloratus</i>	1	1	0	1	0
		<i>Rh. gertrudae</i>	50	27	23	50	0

* Bela-bela farm, Limpopo province.

6.3.3 *Rickettsia* species phylogenetic analysis

6.3.3.1 Citrate synthase gene (*gltA*)

The *Rickettsia gltA* sequences detected in nine (9) ticks (i.e. five (5) *Rh. simus* and four (4) *H. rufipes*) ticks were aligned with representative *Rickettsia* species available on Genbank as per Table 6.4 as shown in Figure 6.8 below. For the purpose of this analysis, only the variability observed in sequences obtained from this study will be described in comparison with the validly published species. The first variation in the sequence is at position (Po) 27 whereby RHSI T50, HYRU 241, HYRU 242, and HYRU 248 have an adenosine (A) base in common with *R. africae*, *R. conorii*, *R. honei*, *R. parkeri*, *R. rickettsii*, *R. sibirica* and *R. slovaca*. At Po 39 HYRU 248 had an A base

where the comparative sequences demonstrated either a thymine (T) or a cytosine (C) in that position, and at Po 49 RHSI T47, RHSI T 55, RHSI T57 and RHSI T58 all had an A in common with *R. massiliae* and *R. rhipicephali*, species that were both first identified in *Rhipicephalus* ticks (Beati and Raoult, 1993, Hayes and Burgdorfer, 1979). For the remainder of the aligned *gltA* sequences, there is consensus between the species, with the exception of several base differences at Po 51, Po 66, Po 118, Po 154, Po 156 and Po's 161-163 as indicated by the stars in the alignments below. At Po 51 RHSI T50 demonstrated a G nucleotide that was not common to any of the other species to which it was compared. Po 66, RHSI T50 had a T base in common with *R. bellii* and *R. helvetica*, whereas the majority of other rickettsias had a C in that position. At Po's 118, 156 and 161-163, HYRU 241 seemed particularly interesting as it demonstrated several mutations distinct from other species compared in this analysis. At Po 154, HYRU 242 demonstrated a G in common only with *R. bellii* in this alignment.

	10	20	30	40	50
<i>R. aeschlimannii</i> (U59722)	G C T A A G G A T A	A A A A T G A T C C	A T T T A G G T T A	A T G G G T T T T G	G T C A T C G T G T
<i>R. africae</i> (U59733)			A		
<i>R. akari</i> (U59717)		G	G	C	
<i>R. australis</i> (U59718)			G	C	
<i>R. bellii</i> (U59716)	A	A A	T	C	
<i>R. canadensis</i> (U59713)	A	G	C	C	C
<i>R. conorii</i> (U59730)			A		
<i>R. felis</i> (AF210692)			G		C
<i>R. helvetica</i> (U59723)			G	A	C
<i>R. honei</i> (U59726)			A		
<i>R. japonica</i> (U59724)					
<i>R. massiliae</i> (U59719)					A
<i>R. montana</i> (U74756)					
<i>R. parkeri</i> (U59732)			A		
<i>R. prowazekii</i> (M17149)	A	G			A
<i>R. rickettsii</i> (U59729)			A		
<i>R. rhipicephali</i> (U59721)					A
<i>R. sibirica</i> (U59734)			A		
<i>R. slovacica</i> (U59725)			A		
<i>R. typhi</i> (U59714)	A	C			A
RHSI T47 GP					A
RHSI T50 GP			A		
RHSI T55 GP					A
RHSI T57 GP					A
RHSI T58 GP					A
HYRU 241 FSP			A		
HYRU 242 FSP			A		
HYRU 247 FSP					
HYRU 248 FSP			A	A	
	60	70	80	90	100
<i>R. aeschlimannii</i> (U59722)	A T A T A A A A A C	T A T G A C C C G C	G T G C C G C A G T	A C T T A A A G A A	A C G T G C A A A G
<i>R. africae</i> (U59733)					
<i>R. akari</i> (U59717)		A	T		
<i>R. australis</i> (U59718)		A			
<i>R. bellii</i> (U59716)		T	G	G	C
<i>R. canadensis</i> (U59713)	C	A	T		T
<i>R. conorii</i> (U59730)					T
<i>R. felis</i> (AF210692)	T	A			T
<i>R. helvetica</i> (U59723)		C	T	A	T
<i>R. honei</i> (U59726)					
<i>R. japonica</i> (U59724)					
<i>R. massiliae</i> (U59719)					
<i>R. montana</i> (U74756)					
<i>R. parkeri</i> (U59732)					
<i>R. prowazekii</i> (M17149)	G				T
<i>R. rickettsii</i> (U59729)			T		
<i>R. rhipicephali</i> (U59721)					
<i>R. sibirica</i> (U59734)					
<i>R. slovacica</i> (U59725)					
<i>R. typhi</i> (U59714)	G	T	T		T
RHSI T47 GP					
RHSI T50 GP	G	T			
RHSI T55 GP					
RHSI T57 GP					
RHSI T58 GP					
HYRU 241 FSP					
HYRU 242 FSP					
HYRU 247 FSP					
HYRU 248 FSP					

[illegible]

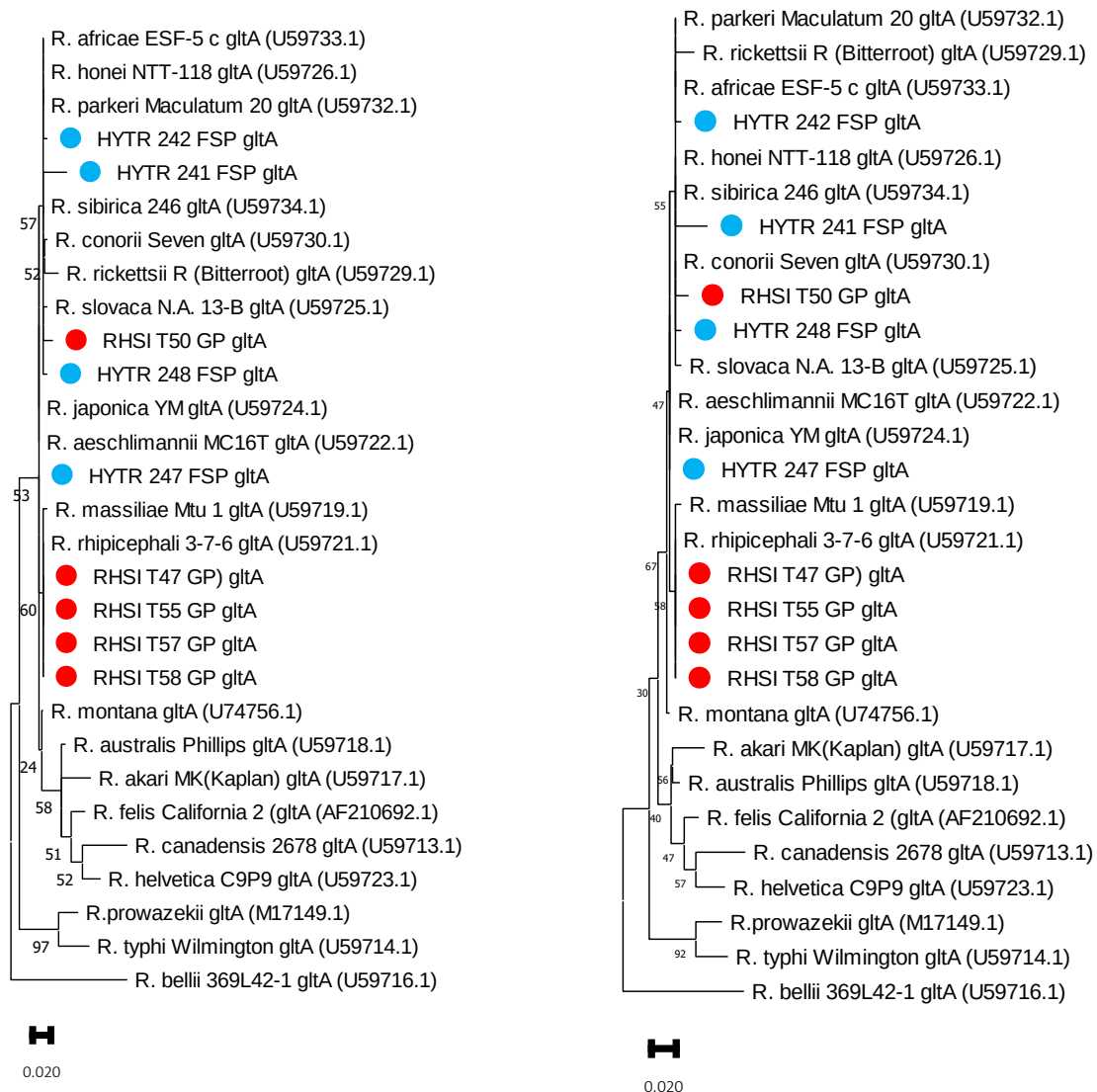


Figure 6.9 Phylogenetic analysis of *Rickettsial citrate synthase (gltA)* gene fragments detected in five (5) *Rhipicephalus simus* (RHSI) and four (4) *Hyalomma rufipes* (HYRU) ticks collected from Gauteng and Free State Provinces respectively. Tree A illustrates evolutionary analysis by Maximum Likelihood method and Tree B infers evolutionary history using the Neighbor-Joining method.

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-730.10) is shown (Figure 6.9-Tree A) with the percentage of trees in which the associated taxa were clustered together is shown below the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. This

analysis involved 29 nucleotide sequences and 200 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) and the optimal tree is shown in Figure 6.9 – Tree B. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown below the branches (Felsenstein, 1985b). The evolutionary distances were computed using the Tamura-Nei method (Tamura and Nei, 1993) and are in the units of the number of base substitutions per site. This analysis involved 29 nucleotide sequences and a total of 197 positions in the final dataset as all positions containing gaps and missing data were eliminated (complete deletion option). Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

It is important to note that several rickettsias detected from the *H. rufipes* ticks (i.e. HYRU 176, HYRU 177, HYRU 248 and HYRU 358) were not included in the phylogenetic trees and sequence alignments as the target area amplified in the initial screening, albeit *gltA*, utilized the ‘in-house’ developed Rick F and Rick R primers instead of the published RpCS.877p and RpCS.1258n ones used for the analyses presented above. Consequently, the DNA was used up during analyses and could not be repeated using published primers. The *gltA* sequences obtained were uploaded onto the BLAST application (<https://blast.ncbi.nlm.nih.gov/Blast>) to determine sequences producing significant alignments. HYRU 176 was found to be closest to several *R. raoultii* and uncultured, unidentified species with 99% coverage and 99.4% of sequence similarity. HYRU 177 was found to be 99.4% similar to *R. sibirica* and 99.1% similar to *R. parkeri*. HYRU 248 demonstrated 98% coverage and 99.1% sequence similarity to various *R. sibirica* isolates, and HYRU 358 demonstrating a 99% coverage and 99.38% sequence similarity to several *R. raoultii* and uncultured, unidentified species as well. Although sequencing results of the *gltA* region cannot effectively differentiate between SFG rickettsias, they do identify our rickettsias as belonging to the SFG.

6.3.2.2 Outer membrane protein A (OmpA)

A 463 bp fragment (common to all compared) of the rickettsial *OmpA* gene was amplified from the five (5) *Rh. simus* ticks, and six (6) *H. rufipes* ticks. These sequences were aligned against twelve (12) validly published representative *Rickettsia* species available on Genbank as per Table 6.4 (see Figure 6.10 below). All nucleotide variability is denoted by blue star in the ruler margins. The variability is described below.

The first position of variability is observed at Po 41, where RHSI T47, RHSI T55, RHSI T57 and RHSI T58 have a T nucleotide in common with *R. massiliae*, a rickettsial species that was first identified in *Rh. turanicus* (Beati and Raoult, 1993). At Po 49, HYRU 176, HYRU 247, HYRU 349 and HYRU 358 had a G nucleotide in common with *R. aeschlimanni*, a rickettsial species initially identified in *H. marginatum* (Beati et al., 1997). There is notable variability in Po's 52, 56 70, 82, 83, 86, and 89-93 amongst the rickettsias compared. RHSI T50 clusters with *R. africae*, *R. conorii*, *R. honei*, and *R. slovaca*. HYRU 177 and HYRU 248 cluster closest to *R. sibirica* sharing an A nucleotide in common at Po 70 (in addition to Po's 52, 56 70, 82, 83, 86, and 89-93), but both lack the T nucleotide at Po 71 demonstrated by *R. sibirica*. RHSI T47, RHSI T55, RHSI T57 and RHSI T58 cluster with *R. massiliae*, whilst HYRU 176, HYRU 247, HYRU 349 and HYRU 358 all clustered with *R. aeschlimannii*.

In the following 50 bases, (Po 100-150) variability was observed at Po's 107, 108, 118, 119, 124, 136, 137, 143, 145-148. Briefly, RHSI T47, RHSI T55, RHSI T57, and RHSI T58 again clustered closest with *R. massiliae* specifically at Po's 118, 124 and 147. HYRU 176, HYRU 247, HYRU349 and HYRU 358 clustered with *R. aeschlimannii* with all nucleotides in common from Po 100 – 150. HYRU 177 and HYRU 248 clustered closest to *R. rickettsii* and *R. sibirica* with a G at Po 118, C at Po 119, G at Po 124, C at Po 143 and GC Po's 146 and 147 respectively. RHSI T50 clustered closest with *R. africae* and *R. honei* with common nucleotides at Po's 108, 118, 124, 137, 143, 146 and 148, but differed at it had a G at Po 107 whereas all species to which it was compared had an A nucleotide in that Po, and instead of a C nucleotide at position 147, RHSI T50 had a T in common with several other rickettsial species.

In Po 151-200, the majority of the variability between the rickettsial species was observed at Po's 159-161, 163, 164 and 187. Three (3) of our rickettsias (i.e. RHSI T50, HYRU 177 and HYRU 248) had a nucleotide pattern of CAATTG in Po's 159-164 and a T at Po 187 which was also observed in several other *Rickettsia* species including *R. africae*, *R. conorii*, *R. honei*, *R. japonica*, *R. parkeri*, *R. sibirica* and *R. slovaca*. The remainder of our rickettsias (i.e. RHSI T47, RHSI T55, RHSI T58, HYRU 176, HYRU 247, HYRU 349 and HYRU358) had a nucleotide sequence of TGGTAT at Po's 159-164 and a C at Po 187 – as sequence common to both *R. aeschlimanii* and *R. massiliae*.

In Po 201-250, RHSI T50, HYRU 177 and HYRU 248 again demonstrated similar nucleotide patterns to the species listed above at Po's 206 and 212, with the exception of RHSI T50 with an A at position 211 – different to the other species but observed in *R. japonica* at that position. The remainder of our rickettsias clustered with *R. aeschlimanii*, *R. australis* and *R. massiliae* in Po's 206, 211-212 and were identical to *R. aeschlimanii* for the rest of the nucleotide sequences to Po 281. In Po's 261-300, variability was observed at Po's 262, 268, 282, 295 and 297. At Po 262 all our rickettsial sequences demonstrated an A nucleotide at that position, except for HYRU177 and HYRU 248 which had a G nucleotide at Po 261 common only to *R. montana*. At Po 268, RHSI T50, HYRU 177 and HYRU 248 demonstrated a G nucleotide common to *R. africae*, *R. conorii*, *R. honei*, *R. japonica*, *R. parkeri*, *R. sibirica* and *R. slovaca*. RHSI T50 however is different to all other species compared as it demonstrated a T at Po 269 instead of the C demonstrated by all the other sequences compared. At Po 282, RHSI T50, HYRU 177 and HYRU 248 demonstrated a T nucleotide in common with *R. aeschlimanii*, *R. africae*, *R. parkeri*, and *R. sibirica*, whilst all our other rickettsias presented a C in that Po common to *R. australis*, *R. conorii*, *R. honei*, *R. japonica*, *R. massiliae*, *R. montana*, *R. rickettsii*, and *R. slovaca*. The nucleotide sequence pattern then reverted to RHSI T50, HYRU 177 and HYRU 248 being similar to *R. africae*, *R. honei*, *R. japonica*, *R. parkeri*, *R. rickettsii*, *R. sibirica* and *R. slovaca* at Po 295-297.

Between Po's 301-350, the nucleotide sequences were fairly conserved and common to all compared species. All our rickettsias demonstrated identical patterns to *R. aeschlimanii*, *R. africae*, *R. conorii*, *R. massiliae* and *R. parkeri*. Some variability in

our rickettsias was observed between Po's 351-400, specifically at Po 352, 359 and 379. Interestingly, the variability at Po 352, i.e. G observed in all the rickettsiae isolated from *Rhipicephalus* ticks except for RHSI T50 instead of the common A observed in all other compared sequences, and at Po 379 where the same demonstrated an A nucleotide instead of the C common to other compared sequences. It is important to note that although this may be distinct from the other species demonstrated in this comparative alignment, it may not be distinct from other *Rickettsia* species that were not included in this analysis e.g. *R. rhipicephali* and others. At Po's 351-400, RHSI T50, HYRU 177 and HYRU 248 demonstrated sequences identical to *R. africae*, *R. parkeri* and *R. sibirica* including an A nucleotide at Po 359 in common, followed by the rest of the sequence (to Po 426) demonstrating no further variability. The final variability observed in the 463 bp fragment was observed at Po's 427, 428, 434, 440, 454 and 449. At Po's 427, 434 and 440, HYRU 177 and HYRU 248 demonstrated G, G and C nucleotides respectively in common with *R. africae*, *R. conorii*, *R. honei*, *R. parkeri*, *R. rickettsii*, *R. sibirica* and *R. slovaca*. RHSI T50, demonstrated a distinctive pattern with G, T, G, T nucleotides at Po's 427, 428, 434 and 440 respectively. The T at Po 428 differs from all other species compared here, but the T at Po 440 is demonstrated by *R. japonica*. The rest of our *Rhipicephalus* rickettsias also demonstrate the G and C at Po's 434 and 440 respectively, but do not demonstrate the G at Po 427, and demonstrate a T at Po 449 which differs to all the rickettsias compared here. Again it is not an exhaustive comparison and these variations would need to be compared with the other rickettsial species not included in this analysis. HYRU 176, HYRU 247, HYRU 349 and HYRU 358 were identical to *R. aeschlimanii* from Po 283 to 463 (i.e. end of the sequence) and are hypothesized to be very closely related to *R. aeschlimanii* which was also first identified in a *Hyalomma* tick (Beati et al., 1997).

	10	20	30	40	50
<i>R. aeschlimannii</i> (U43800)	A T A A T G C T G A	G T A G T A G C G G	G G C A C T C G G T	G T T G C T G	C A G G T G T T G T
<i>R. africana</i> (U43790)	.	.	.	.	.
<i>R. australis</i> (AF149108)	.	.	T G G . C	.	G A .
<i>R. conorii</i> (U43794)	.	.	.	A T T T	.
<i>R. conorii</i> (U43806)	.	.	.	A T T T	.
<i>R. honei</i> (U43809)	.	.	T	.	.
<i>R. japonica</i> (U43795)	.	T	.	.	.
<i>R. massiliae</i> (U43799)	.	.	T	.	T A .
<i>R. montana</i> (U43801)	.	.	T	T	G A .
<i>R. parkeri</i> (U43802)	.	.	.	.	.
<i>R. rickettsii</i> (U43804)	.	.	.	A	.
<i>R. sibirica</i> (U43807)	.	.	.	.	.
<i>R. slovacica</i> (U43808)	.	.	.	.	.
RHSI T47 (GP)	.	.	.	.	T A .
RHSI T50 (GP)	.	.	.	.	.
RHSI T55 (GP)	.	.	.	.	T A .
RHSI T57 (GP)	.	.	.	.	T A .
RHSI T58 (GP)	.	.	.	.	T A .
HYRU 176 (FSP)	.	.	.	.	.
HYRU 177 (FSP)	.	.	.	.	.
HYRU 247 (FSP)	.	.	.	.	.
HYRU 248 (FSP)	.	.	.	.	A
HYRU 349 (FSP)	.	.	.	.	.
HYRU 358 (FSP)	.	.	.	.	.
	60	70	80	90	100
<i>R. aeschlimannii</i> (U43800)	T T C T A A T A A T	A A T A A T G C A G	C A T T T A G T G A	T C T T G C T G C T	G C C A A T A A T T
<i>R. africana</i> (U43790)	G C	.	.	A A T G C	A A T
<i>R. australis</i> (AF149108)	.	G T	.	G T	.
<i>R. conorii</i> (U43794)	G C	.	.	A A T G C	A A T
<i>R. conorii</i> (U43806)	G C	.	.	A A T G C	A A T
<i>R. honei</i> (U43809)	G C	.	.	A A T G C	A A T
<i>R. japonica</i> (U43795)	G C	G	.	G A	.
<i>R. massiliae</i> (U43799)	.	.	.	.	T
<i>R. montana</i> (U43801)	.	G	.	T G	.
<i>R. parkeri</i> (U43802)	G C	.	.	G A T A A C	A A T
<i>R. rickettsii</i> (U43804)	G C	.	A	A A T G C	A A T
<i>R. sibirica</i> (U43807)	G C	A	T	A A T G C	A A T
<i>R. slovacica</i> (U43808)	G C	.	.	A A T G C	A A T
RHSI T47 (GP)	.	.	.	.	T
RHSI T50 (GP)	G C	.	.	A A T A C	A A T
RHSI T55 (GP)	.	.	.	.	T
RHSI T57 (GP)	.	.	.	.	T
RHSI T58 (GP)	.	.	.	.	T
HYRU 176 (FSP)	.	.	.	.	.
HYRU 177 (FSP)	G C	A	.	A A T G C	A A T
HYRU 247 (FSP)	.	.	.	.	.
HYRU 248 (FSP)	G C	A	.	A A T G C	A A T
HYRU 349 (FSP)	.	.	.	.	.
HYRU 358 (FSP)	.	.	.	.	.
	110	120	130	140	150
<i>R. aeschlimannii</i> (U43800)	G G A A T G A T A T	A A C G G C T G A A	G G G A T A G C T A	A T G G T A C T C C	T G T T G A T G G T
<i>R. africana</i> (U43790)	.	G	G	.	C G C A .
<i>R. australis</i> (AF149108)	.	G	A G C T	.	C C
<i>R. conorii</i> (U43794)	.	G	G	.	C C
<i>R. conorii</i> (U43806)	.	G	G	.	C G C
<i>R. honei</i> (U43809)	.	G	G	.	C G C A
<i>R. japonica</i> (U43795)	.	C	A G	.	C G
<i>R. massiliae</i> (U43799)	.	.	C	.	.
<i>R. montana</i> (U43801)	.	G	T	G	C C
<i>R. parkeri</i> (U43802)	G	G	C	G	C G C A
<i>R. rickettsii</i> (U43804)	.	G	C	.	C G C
<i>R. sibirica</i> (U43807)	.	G	C	.	C G C
<i>R. slovacica</i> (U43808)	.	G	G	.	C G C A
RHSI T47 (GP)	.	.	C	.	.
RHSI T50 (GP)	.	G G	C	.	C G A .
RHSI T55 (GP)	.	.	C	.	.
RHSI T57 (GP)	.	.	C	.	.
RHSI T58 (GP)	.	.	C	.	.
HYRU 176 (FSP)	.	.	.	.	.
HYRU 177 (FSP)	.	G	C	G	C G C
HYRU 247 (FSP)	.	.	.	.	.
HYRU 248 (FSP)	.	G	C	G	C G C
HYRU 349 (FSP)	.	.	.	.	.
HYRU 358 (FSP)	.	.	.	.	.

	160	170	180	190	200
<i>R. aeschlimannii</i> (U43800)	CCTCAAAATG	GTATGGCATT	TACTTACGGT	GGTGATCATA	CTATCACTGC
<i>R. africana</i> (U43790)	CAATG			T	
<i>R. australis</i> (AF149108)	GCAAGA			A	
<i>R. conorii</i> (U43794)	CAATG			T	
<i>R. conorii</i> (U43806)	CAATG			T	
<i>R. honei</i> (U43809)	CAATG			T	
<i>R. japonica</i> (U43795)	CAATG			T	
<i>R. massiliae</i> (U43799)					
<i>R. montana</i> (U43801)	G G C A A G				
<i>R. parkeri</i> (U43802)	CAATG			T	
<i>R. rickettsii</i> (U43804)	CAATG			T	G
<i>R. sibirica</i> (U43807)	CAATG			T	
<i>R. slovacica</i> (U43808)	CAATG			T	
RHSI T47 (GP)					
RHSI T50 (GP)	CAATG			T	
RHSI T55 (GP)					
RHSI T57 (GP)					
RHSI T58 (GP)					
HYRU 176 (FSP)					
HYRU 177 (FSP)	CAATG			T	
HYRU 247 (FSP)					
HYRU 248 (FSP)	CAATG			T	
HYRU 349 (FSP)					
HYRU 358 (FSP)					
	210	220	230	240	250
<i>R. aeschlimannii</i> (U43800)	AGATGAAGCC	GGTCGTATTA	TTACGGCTAT	AAATGTTGCG	GGTACTACTC
<i>R. africana</i> (U43790)	C	A		A	
<i>R. australis</i> (AF149108)				A	
<i>R. conorii</i> (U43794)	T	A A			A
<i>R. conorii</i> (U43806)	T	A A			A
<i>R. honei</i> (U43809)	C	A			
<i>R. japonica</i> (U43795)	T T T	AA T	A		AA A
<i>R. massiliae</i> (U43799)					
<i>R. montana</i> (U43801)	A T				
<i>R. parkeri</i> (U43802)	C	A			
<i>R. rickettsii</i> (U43804)	C	A	A		
<i>R. sibirica</i> (U43807)	C T	A			
<i>R. slovacica</i> (U43808)	C	A			
RHSI T47 (GP)					
RHSI T50 (GP)	C	AA			
RHSI T55 (GP)					
RHSI T57 (GP)					
RHSI T58 (GP)					
HYRU 176 (FSP)					
HYRU 177 (FSP)	C	A			
HYRU 247 (FSP)					
HYRU 248 (FSP)	C	A			
HYRU 349 (FSP)					
HYRU 358 (FSP)					
	260	270	280	290	300
<i>R. aeschlimannii</i> (U43800)	CCGTAGGTC	AAATATTACT	CAAAATACCG	TTGTTGGTTC	GATTGTGACG
<i>R. africana</i> (U43790)		G			A A
<i>R. australis</i> (AF149108)	C	G	A	G C C	T T A
<i>R. conorii</i> (U43794)	A	G		C	A
<i>R. conorii</i> (U43806)	A	G		C	A
<i>R. honei</i> (U43809)		G G		C	A A
<i>R. japonica</i> (U43795)		G		C	A A
<i>R. massiliae</i> (U43799)					
<i>R. montana</i> (U43801)	T	G		C	
<i>R. parkeri</i> (U43802)		G			A A
<i>R. rickettsii</i> (U43804)		G	T	C	A A
<i>R. sibirica</i> (U43807)		G		C	A A
<i>R. slovacica</i> (U43808)		G		C	A A
RHSI T47 (GP)		G T		C	A A
RHSI T50 (GP)				C	
RHSI T55 (GP)				C	
RHSI T57 (GP)				C	
RHSI T58 (GP)				C	
HYRU 176 (FSP)		G G			A A
HYRU 177 (FSP)					
HYRU 247 (FSP)					
HYRU 248 (FSP)		G G		C	A A
HYRU 349 (FSP)				C	
HYRU 358 (FSP)				C	

	310	320	330	340	350
<i>R. aeschlimannii</i> (U43800)	G G A G G T A A C T	T G T T G C C T G T	T A C T A T T A C T	G C C G G C A A A A	G C T T A A C T T T
<i>R. africana</i> (U43790)					
<i>R. australis</i> (AF149108)	. T C	 A	 C G	. A	
<i>R. conorii</i> (U43794)					
<i>R. conorii</i> (U43806)					
<i>R. honei</i> (U43809)	A				
<i>R. japonica</i> (U43795)					
<i>R. massiliae</i> (U43799)					
<i>R. montana</i> (U43801)					
<i>R. parkeri</i> (U43802)					
<i>R. rickettsii</i> (U43804)	A A				
<i>R. sibirica</i> (U43807)					
<i>R. slovaca</i> (U43808)	A				
RHSI T47 (GP)					
RHSI T50 (GP)					
RHSI T55 (GP)					
RHSI T57 (GP)					
RHSI T58 (GP)					
HYRU 176 (FSP)					
HYRU 177 (FSP)					
HYRU 247 (FSP)					
HYRU 248 (FSP)					
HYRU 349 (FSP)					
HYRU 358 (FSP)					
	★	360	370	380	390
<i>R. aeschlimannii</i> (U43800)	A A A C G G T A C T	A A T G C T G T T G	C T G C A A A T C A	T G G T T T T G A T	G C T C C T G C C G
<i>R. africana</i> (U43790)					
<i>R. australis</i> (AF149108)	. C A	. . G . . A	 G		. . . G . . . T .
<i>R. conorii</i> (U43794)					
<i>R. conorii</i> (U43806)					
<i>R. honei</i> (U43809)					
<i>R. japonica</i> (U43795)	. . . T . . . A				
<i>R. massiliae</i> (U43799)					
<i>R. montana</i> (U43801)					
<i>R. parkeri</i> (U43802)					
<i>R. rickettsii</i> (U43804)	. . . T . . . A				
<i>R. sibirica</i> (U43807)					
<i>R. slovaca</i> (U43808)					
RHSI T47 (GP)	. G				
RHSI T50 (GP)					
RHSI T55 (GP)	. G				
RHSI T57 (GP)	. G				
RHSI T58 (GP)	. G				
HYRU 176 (FSP)					
HYRU 177 (FSP)					
HYRU 247 (FSP)					
HYRU 248 (FSP)					
HYRU 349 (FSP)					
HYRU 358 (FSP)					
	410	420	430	440	450
<i>R. aeschlimannii</i> (U43800)	A T A A T T A T A C	A G G T T T A G G A	A A T A T A A C T T	T A G A G G G A G A	G A A T G C T G C A
<i>R. africana</i> (U43790)					
<i>R. australis</i> (AF149108)		. G	G C . . . T G . C C	. . G C . . T . C	C . C . . A . G C
<i>R. conorii</i> (U43794)					
<i>R. conorii</i> (U43806)					
<i>R. honei</i> (U43809)	. . . G				
<i>R. japonica</i> (U43795)					
<i>R. massiliae</i> (U43799)					
<i>R. montana</i> (U43801)					
<i>R. parkeri</i> (U43802)					
<i>R. rickettsii</i> (U43804)					
<i>R. sibirica</i> (U43807)					
<i>R. slovaca</i> (U43808)					
RHSI T47 (GP)					
RHSI T50 (GP)					
RHSI T55 (GP)					
RHSI T57 (GP)					
RHSI T58 (GP)					
HYRU 176 (FSP)					
HYRU 177 (FSP)					
HYRU 247 (FSP)					
HYRU 248 (FSP)					
HYRU 349 (FSP)					
HYRU 358 (FSP)					

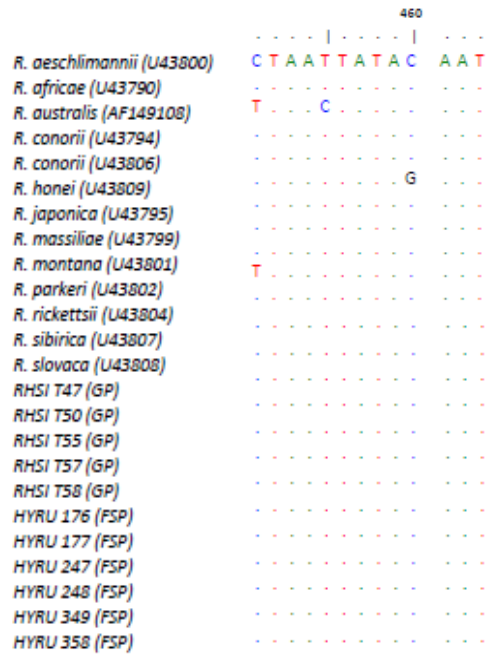
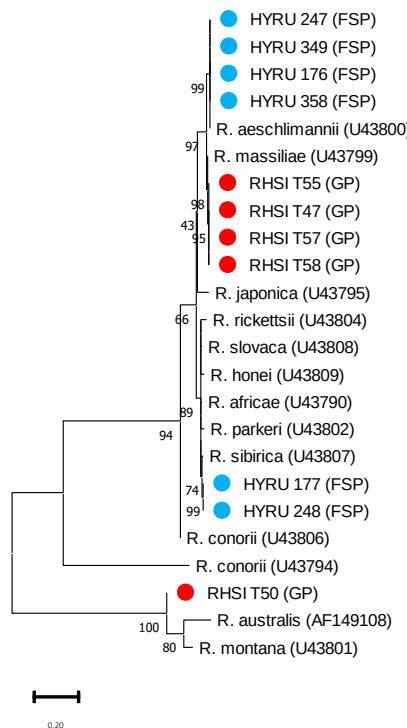


Figure 6.10 Sequence data obtained from the amplification of *Rickettsia* OmpA fragments identified in five (5) *Rh. simus* ticks (i.e. denoted by the R. HSI prefixes), six (6) *H. rufipes* ticks (i.e. denoted by the HYRU prefixes) aligned with the *Rickettsia* species as previously published (Fournier et al., 2003). A total of 463 bp positions were aligned and all smaller products were excluded from this analysis.

Tree C: Maximum Likelihood method



Tree D: Neighbor-Joining method

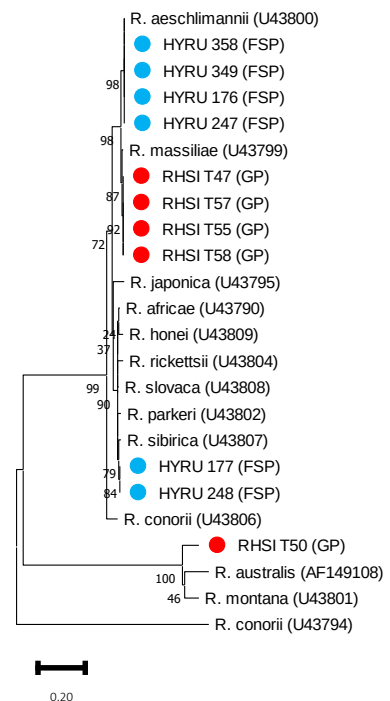


Figure 6.11 Phylogenetic analysis of *Rickettsial* Outer Membrane Protein A (OmpA) fragments detected in five (5) *Rh. simus* (R. HSI) collected from Gauteng and six (6) *H. rufipes* ticks collected from the Free State Province. Tree C illustrates evolutionary analysis by Maximum Likelihood method and Tree D infers evolutionary history using the Neighbor-Joining method.

Using the Maximum Likelihood method and Tamura-Nei model, the evolutionary history was inferred (Tamura and Nei, 1993). The percentage of trees in which the associated taxa are clustered together is shown beneath the branches and tree C demonstrates the highest log likelihood (-2801.01). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. The analysis involved 24 nucleotide sequences, with 1st+2nd+3rd+Noncoding codon positions included. There were a total of 463 positions in the final dataset and evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

The Neighbor-Joining method was used to infer the evolutionary history (Saitou and Nei, 1987). The optimal tree is shown in Figure 6.11 - Tree D and the percentage of replicate trees in which the associated taxa are clustered together in the bootstrap test (1,000 replicates) are shown below the branches (Felsenstein, 1985b). The evolutionary distances were computed using the Tamura-Nei method (Tamura and Nei, 1993) and are in the units of the number of base substitutions per site. The analysis took into account 24 nucleotide sequences, including 1st+2nd+3rd+Noncoding codon positions. All positions containing gaps and missing data were eliminated (complete deletion option) resulting in a total of 457 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

6.3.2.3 Outer Membrane Protein B (OmpB)

The *OmpB* region was demonstrably interesting when our rickettsias were aligned with the gene, the same primers were used for all samples, but the region amplified from the *Hyalomma* rickettsias differed from those amplified from the *Rhipicephalus* ones. The alignment provided (i.e. Figure 6.12) demonstrates the alignment positions for both the *Hyalomma* rickettsias and the *Rhipicephalus* ones compared to a 1,214 bp portion of the *OmpB* gene. A blue star in the upper ruler section is used to highlight the nucleotide variations in our rickettsias when compared to validly published *Rickettsia* species. The areas where our rickettsias were not aligned are demonstrated by greyed out blocks. Our *Hyalomma* tick rickettsias for which an *OmpB*

region was amplified (i.e. HYRU176 and HYRU248), were aligned between Po 1 and Po's 709 and 710 respectively, whereas our *Rhipicephalus rickettsias* (i.e. RHSI T47, RHSI T50, RHSI T57 and RHSI T58) were aligned at Po's 823, 808, 832 and 823 respectively to Po 1214 (end).

Between Po's 1-50, the nucleotide sequences demonstrated clear similarities (i.e. 100 % congruency) between HYRU 176 and *R. aeschlimannii* and HYRU 248 and *R. sibirica*. In Po's 51-100, HYRU 176 continued to demonstrate congruency with *R. aeschlimannii* as well as additional species demonstrating the same nucleotide sequence, namely *R. conorii*, *R. rickettsii*, *R. rhipicephali* and *R. slovaca*, and HYRU 248 continued to demonstrate congruency with *R. sibirica*, as well as *R. africae* and *R. parkeri*. The region between Po's 101-150 was fairly conserved for most of the species compared, with HYRU 248 demonstrating only one (1) nucleotide (a T at Po 117 in common with *R. prowazekii*) difference from the majority of species compared.

In Po's 151-200, HYRU 176 demonstrated 100 % congruency with *R. aeschlimannii* and *R. rhipicephali*. HYRU 248 demonstrated two (2) nucleotide differences at Po's 157 (T instead of C) and 160 (G instead of A) which were common to *R. conorii*, *R. parkeri*, *R. rickettsii* and *R. sibirica*. HYRU 176 was again 100 % congruent with *R. aeschlimannii* in Po's 201-250, whereas HYRU 248 demonstrated nucleotide variability (i.e. G, T, C and G at Po's 202, 217, 221 and 230 respectively) in common with *R. africae*, *R. parkeri* and *R. sibirica*. Between Po's 251-300, nucleotide variability was demonstrated at Po's 253 and 272. Based on these variations, HYRU 176 clustered with *R. aeschlimannii*, *R. massiliae* and *R. rhipicephali*, whereas HYRU 248 clustered with *R. africae*, *R. conorii*, *R. honei*, *R. parkeri*, *R. rickettsii*, *R. sibirica* and *R. slovaca*. In Po's 301-350, HYRU 176 was 100 % congruent with *R. aeschlimannii* and *R. parkeri*, whereas HYRU 248 demonstrating 100 % congruency with *R. sibirica* with a C nucleotide in common with the species at Po 329. Po's 351-450 demonstrated some variability between species compared, however HYRU 176 remained 100 % consistent with *R. aeschlimannii*, and HYRU 248 similar to *R. africae*, *R. conorii*, and *R. sibirica* in Po's 351-400, and similar to *R. africae*, *R. parkeri* and *R. sibirica* in Po's 401-450.

In the next 150 nucleotides, i.e. Po's 451-600, HYRU 176 sustained its 100 % consistency with *R. aeschlimannii*, whilst HYRU 248 shared nucleotide sequences common to many of the other species compared in the alignment. In Po 605, HYRU 176 demonstrated a C nucleotide different to *R. aeschlimannii*, but in common with *R. massiliae* and *R. rhipicephali*, with the remainder of the region (i.e. Po's 601-650) demonstrating a sequence identical to *R. aeschlimannii*, *R. massiliae* and *R. rhipicephali*. HYRU 248 maintained its similarity to *R. africae*, *R. conorii*, *R. parkeri* and *R. sibirica*, particularly in Po's 605, 611-613, and 623 where some nucleotide variability was observed. Finally, in Po's 651-709, it was revealed that HYRU 176 had a nucleotide sequence identical to *R. aeschlimannii* and *R. japonica*, whereas HYRU 248 did not cluster specifically with any one (1) species or another in the region, to its end at Po 710. Between Po 710 and 808, none of our rickettsias were aligned.

At Po 808, the first of the *Rhipicephalus*-derived rickettsias (i.e. RHSI T50) aligned producing a 406 bp product alignment. At Po. 823, RHSI T47 and RHSI T58 (391 bp) began to align, and at Po 832, RHSI T57 aligned to produce a 382 bp alignment. At Po 847, RHSI T50 demonstrated a nucleotide difference from its counterparts with a T (common to *R. montanensis*, *R. prowazekii* and *R. typhi*) nucleotide instead of the C observed for all the other compared species. In the region Po's 851-900, areas of variability were observed at Po's 877, 882, 895 and 899. At Po 877, RHSI T47, T57 and T58 all demonstrated a G nucleotide in common with *R. montanensis*, whereas all the other species compared had an A in that position. RHSI T50 is distinct from the other *Rhipicephalus*-derived rickettsias due to its variability at Po 882 (i.e. C instead of a T), Po 895 with the only T observed in that position (all others compared demonstrated a C in that Po) and a G at Po 899. Between Po's 901-983, are four (4) RHSI rickettsiae which were identical to each other and the majority of other *Rickettsia* species, however, at Po's 984 and 987, RHSI T50 demonstrated an A and G respectively, setting it apart from the other three (3) RHSI rickettsias. Its nucleotide sequence between Po's 951-1000 was identical to *R. africae*, *R. honei* and *R. parkeri*. At Po 995, RHSI T47, T57 and T58 demonstrated a T nucleotide instead of the C that was visible in the other species in the alignment, and taking into consideration the entire 50 bp region (i.e. 951-1000), were found to be 100 % identical to *R. rhipicephali*, *R. massiliae* and *R. japonica*. In the next 50 bp region (i.e. 1001-1050), RHSI T50 clustered with *R. africae*, *R. parkeri*, *R. sibirica* and *R. slovaca* demonstrating identical

nucleotide sequences with a T at Po 1001, a G at Po 1029 and an A at Po 1032. The other RHSI rickettsias clustered only with *R. massiliae* in the region between 1001-1100 with 100 % consistency throughout with an A nucleotide at Po 1095 setting them apart from the other species compared. In Po's 1051-1100, RHSI T50 was fairly conserved demonstrating a sequence common to several other species compared.

The final 164 nucleotides were also fairly conserved with the exception of several interesting variations observed that set our RHSI rickettsias apart from counterparts to which they were aligned. RHSI T47, T57 and T58 had a C at Po 1130 unlike all other species to which it was compared. RHSI T50 demonstrated an A at Po 1143, distinct from all other species compared which had a G in that Po. It then was observed to have a T at Po 1152, and an A at Po 1169 which again were decidedly different to the other species in the comparative analysis. RHSI T47, T57 and T58 demonstrated a three (3) bp insertion, 'CAA' at Po 1156-1158, with no further interesting anomalies detected thereafter.

	10	20	30	40	50
<i>R. aeschlimannii</i> (AF123705)	A T G G C T T A G G	C A C A G G T A T T	G G C T C T T C A C	A G T T A A A G C A	A G T A A C G T T T
<i>R. africae</i> (AF123706)			G	C	
<i>R. akari</i> (AF123707)	C	T	T G T	G G C	A T A G C
<i>R. australis</i> (AF123709)	C	T	T G T	A G C A C	
<i>R. conorii</i> (AF123721)			G	A	C
<i>R. conorii</i> (AF123726)			A	A	C
<i>R. felis</i> (AF210695)	C	T	T T	A G A C	T A G C G A
<i>R. helvetica</i> (AF123725)	C A	T	T T T	A C	T A A C A
<i>R. honei</i> (AF123724)			C A	A	C
<i>R. japonica</i> (AF123713)			G	A	C
<i>R. massiliae</i> (AF123714)			G		
<i>R. montanensis</i> (AF123716)		A A G C	A	G	C
<i>R. parkeri</i> (AF123717)			G	A	C
<i>R. prowazekii</i> (AF123718)	T	T T T	A G A	T A G C	A A G A
<i>R. rickettsii</i> (X16353)			G	A	C
<i>R. rhipicephali</i> (AF123719)			G		
<i>R. sibirica</i> (AF123722)			T G	A	C
<i>R. slovacica</i> (AF123723)			A	C	C
<i>R. typhi</i> (L04661)	T	T T T	A G A	T G A C	
HYRU 176 (FSP) OmpB					
HYTR 248 (FSP) OmpB			T G	A	C
RHSI T47 (GP) OmpB					
RHSI T50 (GP) OmpB					
RHSI T57 (GP) OmpB					
RHSI T58 (GP) OmpB					

	60	70	80	90	100
<i>R. aeschlimannii</i> (AF123705)	A C T A C A G A C T	A T A A C A A C T T	A G G T A A T A T T	A T T G C A A C T A	A C G C A A C A A T
<i>R. africae</i> (AF123706)		T			
<i>R. akari</i> (AF123707)	G		G	G C	T C
<i>R. australis</i> (AF123709)		T	G	C	T
<i>R. conorii</i> (AF123721)					
<i>R. conorii</i> (AF123726)					
<i>R. felis</i> (AF210695)			G	C	
<i>R. helvetica</i> (AF123725)	A		G		C
<i>R. honei</i> (AF123724)					
<i>R. japonica</i> (AF123713)					A
<i>R. massiliae</i> (AF123714)				G	
<i>R. montanensis</i> (AF123716)					A
<i>R. parkeri</i> (AF123717)		T			
<i>R. prowazekii</i> (AF123718)	T		G	A	T T
<i>R. rickettsii</i> (X16353)					
<i>R. rhipicephali</i> (AF123719)					
<i>R. sibirica</i> (AF123722)		T			
<i>R. slovacica</i> (AF123723)					
<i>R. typhi</i> (L04661)	T		G		T
HYRU 176 (FSP) OmpB					
HYTR 248 (FSP) OmpB		T			
RHSI T47 (GP) OmpB					
RHSI T50 (GP) OmpB					
RHSI T57 (GP) OmpB					
RHSI T58 (GP) OmpB					

	110	120	130	140	150
<i>R. aeschlimannii</i> (AF123705)	T A A T G A C G G T	G T A A C T G T T A	C T A C A G G C G G	T A T A G C C G	
<i>R. africae</i> (AF123706)					
<i>R. akari</i> (AF123707)			G	T	
<i>R. australis</i> (AF123709)			T	G	T
<i>R. conorii</i> (AF123721)					
<i>R. conorii</i> (AF123726)				T	
<i>R. felis</i> (AF210695)			T	G	
<i>R. helvetica</i> (AF123725)	T				
<i>R. honei</i> (AF123724)					
<i>R. japonica</i> (AF123713)				C A	G G G G T A T A G
<i>R. massiliae</i> (AF123714)					
<i>R. montanensis</i> (AF123716)					
<i>R. parkeri</i> (AF123717)					
<i>R. prowazekii</i> (AF123718)	T A	C	A	A	
<i>R. rickettsii</i> (X16353)					
<i>R. rhipicephali</i> (AF123719)					
<i>R. sibirica</i> (AF123722)					
<i>R. slovacica</i> (AF123723)					
<i>R. typhi</i> (L04661)	A	A C	A		
HYRU 176 (FSP) OmpB					
HYTR 248 (FSP) OmpB	T				
RHSI T47 (GP) OmpB					
RHSI T50 (GP) OmpB					
RHSI T57 (GP) OmpB					
RHSI T58 (GP) OmpB					

	160	170	180	190	200
<i>R. aeschlimannii</i> (AF123705)	..- 	..- 	..- 	..- 	..-
<i>R. africana</i> (AF123706)	..- G A A C A G A	..- T T T T G A C G G T	..- A A A A T T A C T C	..- T T G G A A G T G T	..- T A A C G G T A A C
<i>R. akari</i> (AF123707)	..-	..-	..-	..-	..-
<i>R. australis</i> (AF123709)	..- . G	..- . C	..- . C	..-	..-
<i>R. conorii</i> (AF123721)	..-	..- . C	..- . C	..-	..-
<i>R. conorii</i> (AF123726)	..-	..- . C	..- . C	..-	..-
<i>R. felis</i> (AF210695)	..-	..- . C	..- . C	..-	..-
<i>R. helvetica</i> (AF123725)	..-	..- . C	..- . C	..-	..-
<i>R. honei</i> (AF123724)	..-	..- . C	..- . C	..-	..-
<i>R. japonica</i> (AF123713)	C C G	..-	..-	..-	..-
<i>R. massiliae</i> (AF123714)	..-	..-	..-	..-	..-
<i>R. montanensis</i> (AF123716)	..-	..-	..-	..-	..-
<i>R. parkeri</i> (AF123717)	..-	..-	..-	..-	..-
<i>R. prowazekii</i> (AF123718)	..- . G	..-	..-	..-	..-
<i>R. rickettsii</i> (X16353)	..-	..-	..-	..-	..-
<i>R. rhipicephali</i> (AF123719)	..-	..-	..-	..-	..-
<i>R. sibirica</i> (AF123722)	..-	..-	..-	..-	..-
<i>R. slovacica</i> (AF123723)	..-	..-	..-	..-	..-
<i>R. typhi</i> (L04661)	..- . G	..-	..-	..-	..-
HYRU 176 (FSP) OmpB	..-	..-	..-	..-	..-
HYTR 248 (FSP) OmpB	..-	..-	..-	..-	..-
RHSI T47 (GP) OmpB	..-	..-	..-	..-	..-
RHSI T50 (GP) OmpB	..-	..-	..-	..-	..-
RHSI T57 (GP) OmpB	..-	..-	..-	..-	..-
RHSI T58 (GP) OmpB	..-	..-	..-	..-	..-

	210	220	230	240	250
<i>R. aeschlimannii</i> (AF123705)	G C T A A T G T A A	G A T T T G C T G A	T G G T A T A T T T	T C T A A T T C T A	C A A G T A T G A T
<i>R. africana</i> (AF123706)	. G				
<i>R. akari</i> (AF123707)		. G	. C A . C . C .		
<i>R. australis</i> (AF123709)			. C C . C . C .		
<i>R. conorii</i> (AF123721)	. G		. C		
<i>R. conorii</i> (AF123726)	. G		. C		
<i>R. felis</i> (AF210695)			. C		
<i>R. helvetica</i> (AF123725)	T	T T	C . . . C . A A C	 C . G . .	
<i>R. honei</i> (AF123724)	. G		. C . . . G . G .		
<i>R. japonica</i> (AF123713)			. C		
<i>R. massiliae</i> (AF123714)			. C		
<i>R. montanensis</i> (AF123716)			. C		
<i>R. parkeri</i> (AF123717)	. G		. C		
<i>R. prowazekii</i> (AF123718)	 C . .	. G	. A . . . C . .	 G . C . .	
<i>R. rickettsii</i> (X16353)	. G		. C		
<i>R. rhipicephali</i> (AF123719)			. C		
<i>R. sibirica</i> (AF123722)	. G		. C		
<i>R. slovacica</i> (AF123723)	. G		. C		
<i>R. typhi</i> (L04661)		A G	C A . A . C . .	 C . C . .	
HYRU 176 (FSP) OmpB					
HYTR 248 (FSP) OmpB	. G		. C		
RHSI T47 (GP) OmpB					
RHSI T50 (GP) OmpB					
RHSI T57 (GP) OmpB					
RHSI T58 (GP) OmpB					

	260	270	280	290	300
<i>R. aeschlimannii</i> (AF123705)	T G T T A C T A C T	A A A G C T A A T A	A C G G T A C T G T	A A C T T A T T T A	G G T A A T G C A T
<i>R. africana</i> (AF123706)	. . G				
<i>R. akari</i> (AF123707)	. . G				
<i>R. australis</i> (AF123709)					
<i>R. conorii</i> (AF123721)	. . G				
<i>R. conorii</i> (AF123726)	. . G				
<i>R. felis</i> (AF210695)					
<i>R. helvetica</i> (AF123725)	. . C . . A G .				
<i>R. honei</i> (AF123724)	. . G				
<i>R. japonica</i> (AF123713)					
<i>R. massiliae</i> (AF123714)					
<i>R. montanensis</i> (AF123716)					
<i>R. parkeri</i> (AF123717)	. . G				
<i>R. prowazekii</i> (AF123718)	 G . .				
<i>R. rickettsii</i> (X16353)	. . G				
<i>R. rhipicephali</i> (AF123719)					
<i>R. sibirica</i> (AF123722)	. . G				
<i>R. slovacica</i> (AF123723)	. . G				
<i>R. typhi</i> (L04661)	 T . .				
HYRU 176 (FSP) OmpB					
HYTR 248 (FSP) OmpB	. . G				
RHSI T47 (GP) OmpB					
RHSI T50 (GP) OmpB					
RHSI T57 (GP) OmpB					
RHSI T58 (GP) OmpB					

	310	320	330	340	350
<i>R. aeschlimannii</i> (AF123705)	T C G T C G G T A A	T A T A G G T G A T	T C A G G T A C T C	C T G T A G C T T C	T G T T A G A T T T
<i>R. africana</i> (AF123706)					
<i>R. akari</i> (AF123707)	A T	 C	 T . A . G . A	A . A	 A G .
<i>R. australis</i> (AF123709)	A T	 C	G . T . A	A	 A G .
<i>R. conorii</i> (AF123721)					
<i>R. conorii</i> (AF123726)					
<i>R. felis</i> (AF210695)	. A	 A G .	 A		 A .
<i>R. helvetica</i> (AF123725)	C T . . A	 C . . A G C	. . T A A . G . . .	T	 A . A .
<i>R. honei</i> (AF123724)	 T		 A		
<i>R. japonica</i> (AF123713)					
<i>R. massiliae</i> (AF123714)	G		 A		
<i>R. montanensis</i> (AF123716)	A G		 A A . . .		
<i>R. parkeri</i> (AF123717)					
<i>R. prowazekii</i> (AF123718)	. A . . T A . . .	 C . . A G	. . T . A		T G T T A G A T T T
<i>R. rickettsii</i> (X16353)					
<i>R. rhipicephali</i> (AF123719)	G		 A A . . .		
<i>R. sibirica</i> (AF123722)					
<i>R. slovaca</i> (AF123723)	. A		 A . . C .		
<i>R. typhi</i> (L04661)	. A	 T . . A G	 A . T . .		
HYRU 176 (FSP) OmpB					
HYTR 248 (FSP) OmpB					
RHSI T47 (GP) OmpB					
RHSI T50 (GP) OmpB					
RHSI T57 (GP) OmpB					
RHSI T58 (GP) OmpB					

	360	370	380	390	400
<i>R. aeschlimannii</i> (AF123705)	A C A G G T A G T G		A T G A T G G T	G C A G G A T T A C	A A G G A A A T A T
<i>R. africana</i> (AF123706)					
<i>R. akari</i> (AF123707)	 A . C		 A		
<i>R. australis</i> (AF123709)	. . G . . . A . .		 A . . G .		
<i>R. conorii</i> (AF123721)					
<i>R. conorii</i> (AF123726)					
<i>R. felis</i> (AF210695)	. T				
<i>R. helvetica</i> (AF123725)	. . G . . . C C . .	C C G	G . T C A T T A	. A . A A	
<i>R. honei</i> (AF123724)					
<i>R. japonica</i> (AF123713)					
<i>R. massiliae</i> (AF123714)					
<i>R. montanensis</i> (AF123716)	. . G	G T A C G G G T A G	T G . . A	 T	
<i>R. parkeri</i> (AF123717)					
<i>R. prowazekii</i> (AF123718)					
<i>R. rickettsii</i> (X16353)					
<i>R. rhipicephali</i> (AF123719)					
<i>R. sibirica</i> (AF123722)					
<i>R. slovaca</i> (AF123723)					
<i>R. typhi</i> (L04661)	. . T . . . A . .		 A G . . .	. . T	 C . .
HYRU 176 (FSP) OmpB					
HYTR 248 (FSP) OmpB					
RHSI T47 (GP) OmpB					
RHSI T50 (GP) OmpB					
RHSI T57 (GP) OmpB					
RHSI T58 (GP) OmpB					

	410	420	430	440	450
<i>R. aeschlimannii</i> (AF123705)	T T A T T C A C A A	G T C A T A G A C T	T T G G T A C T T A	T G A C T T A G G T	A T T T T A A A T T
<i>R. africana</i> (AF123706)					
<i>R. akari</i> (AF123707)		 C G . . T		. A C T . . . A C	G
<i>R. australis</i> (AF123709)		 C . . . T	 G	 T . . A C	G
<i>R. conorii</i> (AF123721)					
<i>R. conorii</i> (AF123726)					
<i>R. felis</i> (AF210695)					
<i>R. helvetica</i> (AF123725)	A T A C .	 C . . C C A . T	 A . G T .	. A A A .	G . . G C T G G G .
<i>R. honei</i> (AF123724)					
<i>R. japonica</i> (AF123713)					
<i>R. massiliae</i> (AF123714)					
<i>R. montanensis</i> (AF123716)					
<i>R. parkeri</i> (AF123717)					
<i>R. prowazekii</i> (AF123718)		A A T	 T	. A . T . . . A C .	 C . .
<i>R. rickettsii</i> (X16353)					
<i>R. rhipicephali</i> (AF123719)					
<i>R. sibirica</i> (AF123722)					
<i>R. slovaca</i> (AF123723)					
<i>R. typhi</i> (L04661)	. C . C	A A T	 A	. A A C .	
HYRU 176 (FSP) OmpB					
HYTR 248 (FSP) OmpB					
RHSI T47 (GP) OmpB					
RHSI T50 (GP) OmpB					
RHSI T57 (GP) OmpB					
RHSI T58 (GP) OmpB					

	460	470	480	490	500
<i>R. aeschlimannii</i> (AF123705)	C T A A T G T A A T	T T T A G G C G G T	G G T A C T A C T G	C T A T T A A C G G	T G C A G T C A A T
<i>R. africana</i> (AF123706)		C		A A A	
<i>R. akari</i> (AF123707)	C	G		A	G
<i>R. australis</i> (AF123709)	C	C		A A	G
<i>R. conorii</i> (AF123721)		C		A A A	
<i>R. conorii</i> (AF123726)		C		A A A	
<i>R. felis</i> (AF210695)	C	C		A A A	G
<i>R. helvetica</i> (AF123725)	G A	T C	A C A	T	A A T A T
<i>R. honei</i> (AF123724)		C		A A A	G
<i>R. japonica</i> (AF123713)		C A		A A A	G
<i>R. massiliae</i> (AF123714)		C		A	
<i>R. montanensis</i> (AF123716)				A	G
<i>R. parkeri</i> (AF123717)		C		A A A	
<i>R. prowazekii</i> (AF123718)	C			T	A A A
<i>R. rickettsii</i> (X16353)	A	C		A A A	G
<i>R. rhipicephali</i> (AF123719)		C		A	
<i>R. sibirica</i> (AF123722)		C		A A A	
<i>R. slovacica</i> (AF123723)		C		A A A	
<i>R. typhi</i> (L04661)	G			T	A G A T G
HYRU 176 (FSP) OmpB					
HYTR 248 (FSP) OmpB		C			A A A
RHSI T47 (GP) OmpB					
RHSI T50 (GP) OmpB					
RHSI T57 (GP) OmpB					
RHSI T58 (GP) OmpB					

	510	520	530	540	550
<i>R. aeschlimannii</i> (AF123705)	C T T C T T A C G A	A T A C T T T A A C	A T T T G C A A G T	G G T A C T T C A A	C A T G G G G A A A
<i>R. africana</i> (AF123706)	A				
<i>R. akari</i> (AF123707)	A	C	C		G
<i>R. australis</i> (AF123709)	A	C	C		C G
<i>R. conorii</i> (AF123721)	G A				
<i>R. conorii</i> (AF123726)	A				
<i>R. felis</i> (AF210695)	A	C	C		G
<i>R. helvetica</i> (AF123725)	G A A	C	A C		G
<i>R. honei</i> (AF123724)	A				
<i>R. japonica</i> (AF123713)	A		G		
<i>R. massiliae</i> (AF123714)	A				
<i>R. montanensis</i> (AF123716)	T G			T	
<i>R. parkeri</i> (AF123717)	A				
<i>R. prowazekii</i> (AF123718)	G A	A T	A		T G
<i>R. rickettsii</i> (X16353)	G	A			
<i>R. rhipicephali</i> (AF123719)	A				
<i>R. sibirica</i> (AF123722)	A				
<i>R. slovacica</i> (AF123723)	A				
<i>R. typhi</i> (L04661)	T G A	A T	A		C
HYRU 176 (FSP) OmpB					
HYTR 248 (FSP) OmpB	A				
RHSI T47 (GP) OmpB					
RHSI T50 (GP) OmpB					
RHSI T57 (GP) OmpB					
RHSI T58 (GP) OmpB					

	560	570	580	590	600
<i>R. aeschlimannii</i> (AF123705)	C A A T A C T T C T	A T T A A A A C T A	C T T T A A C A T T	A G C A A A C G G T	A A T A T A G G T C
<i>R. africana</i> (AF123706)		G		T	A
<i>R. akari</i> (AF123707)		G	G		G
<i>R. australis</i> (AF123709)		G	G		G
<i>R. conorii</i> (AF123721)		G			A
<i>R. conorii</i> (AF123726)		G			A
<i>R. felis</i> (AF210695)		G	G		
<i>R. helvetica</i> (AF123725)	T	C G C A	G	T	
<i>R. honei</i> (AF123724)		G		A	
<i>R. japonica</i> (AF123713)	T	G			
<i>R. massiliae</i> (AF123714)			G		
<i>R. montanensis</i> (AF123716)		G		G	
<i>R. parkeri</i> (AF123717)		G			A
<i>R. prowazekii</i> (AF123718)	T	G T A	G A T G	T G	
<i>R. rickettsii</i> (X16353)		G			
<i>R. rhipicephali</i> (AF123719)			G		
<i>R. sibirica</i> (AF123722)		G			A
<i>R. slovacica</i> (AF123723)		G			
<i>R. typhi</i> (L04661)	T C	C G T A	A A C G	T	G
HYRU 176 (FSP) OmpB					
HYTR 248 (FSP) OmpB	G	G			A
RHSI T47 (GP) OmpB					
RHSI T50 (GP) OmpB					
RHSI T57 (GP) OmpB					
RHSI T58 (GP) OmpB					

	610	620	630	640	650
<i>R. aeschlimannii</i> (AF123705)	★	★ ★ ★	★		
<i>R. africana</i> (AF123706)					
<i>R. akari</i> (AF123707)					
<i>R. australis</i> (AF123709)					
<i>R. conorii</i> (AF123721)					
<i>R. conorii</i> (AF123726)					
<i>R. felis</i> (AF210695)					
<i>R. helvetica</i> (AF123725)					
<i>R. honei</i> (AF123724)					
<i>R. japonica</i> (AF123713)					
<i>R. massiliae</i> (AF123714)					
<i>R. montanensis</i> (AF123716)					
<i>R. parkeri</i> (AF123717)					
<i>R. prowazekii</i> (AF123718)					
<i>R. rickettsii</i> (X16353)					
<i>R. rhipicephali</i> (AF123719)					
<i>R. sibirica</i> (AF123722)					
<i>R. slovacica</i> (AF123723)					
<i>R. typhi</i> (L04661)					
HYRU 176 (FSP) OmpB					
HYTR 248 (FSP) OmpB					
RHSI T47 (GP) OmpB					
RHSI T50 (GP) OmpB					
RHSI T57 (GP) OmpB					
RHSI T58 (GP) OmpB					

	660	670	680	690	700
<i>R. aeschlimannii</i> (AF123705)	★				
<i>R. africana</i> (AF123706)					
<i>R. akari</i> (AF123707)					
<i>R. australis</i> (AF123709)					
<i>R. conorii</i> (AF123721)					
<i>R. conorii</i> (AF123726)					
<i>R. felis</i> (AF210695)					
<i>R. helvetica</i> (AF123725)					
<i>R. honei</i> (AF123724)					
<i>R. japonica</i> (AF123713)					
<i>R. massiliae</i> (AF123714)					
<i>R. montanensis</i> (AF123716)					
<i>R. parkeri</i> (AF123717)					
<i>R. prowazekii</i> (AF123718)					
<i>R. rickettsii</i> (X16353)					
<i>R. rhipicephali</i> (AF123719)					
<i>R. sibirica</i> (AF123722)					
<i>R. slovacica</i> (AF123723)					
<i>R. typhi</i> (L04661)					
HYRU 176 (FSP) OmpB					
HYTR 248 (FSP) OmpB					
RHSI T47 (GP) OmpB					
RHSI T50 (GP) OmpB					
RHSI T57 (GP) OmpB					
RHSI T58 (GP) OmpB					

	710	720	730	740	750
<i>R. aeschlimannii</i> (AF123705)					
<i>R. africana</i> (AF123706)					
<i>R. akari</i> (AF123707)					
<i>R. australis</i> (AF123709)					
<i>R. conorii</i> (AF123721)					
<i>R. conorii</i> (AF123726)					
<i>R. felis</i> (AF210695)					
<i>R. helvetica</i> (AF123725)					
<i>R. honei</i> (AF123724)					
<i>R. japonica</i> (AF123713)					
<i>R. massiliae</i> (AF123714)					
<i>R. montanensis</i> (AF123716)					
<i>R. parkeri</i> (AF123717)					
<i>R. prowazekii</i> (AF123718)					
<i>R. rickettsii</i> (X16353)					
<i>R. rhipicephali</i> (AF123719)					
<i>R. sibirica</i> (AF123722)					
<i>R. slovacica</i> (AF123723)					
<i>R. typhi</i> (L04661)					
HYRU 176 (FSP) OmpB					
HYTR 248 (FSP) OmpB					
RHSI T47 (GP) OmpB					
RHSI T50 (GP) OmpB					
RHSI T57 (GP) OmpB					
RHSI T58 (GP) OmpB					

	760	770	780	790	800
<i>R. aeschlimannii</i> (AF123705)	T T C C T A A C T T	T G C T G T A A C C	G G A A G T A A T C	G T T T C G T A A A	T T A C G G T T T A
<i>R. africana</i> (AF123706)	G . . . C	. . T			
<i>R. akari</i> (AF123707)	G T . . .			 T . . .	 C . . .
<i>R. australis</i> (AF123709)	G				 T . . .
<i>R. conorii</i> (AF123721)	G . . . C	. . T			
<i>R. conorii</i> (AF123726)	G . . . C	. . T			
<i>R. felis</i> (AF210695)	G			 T . . .	
<i>R. helvetica</i> (AF123725)	A				
<i>R. honei</i> (AF123724)	G . . . C				
<i>R. massiliae</i> (AF123714)	G				
<i>R. montanensis</i> (AF123716)	A G				
<i>R. parkeri</i> (AF123717)	G . . . C	. . T			 T . . .
<i>R. prowazekii</i> (AF123718)	C	 A . . .	 A . . .	T	A . . T . A A C .
<i>R. rickettsii</i> (X16353)	G . . . C				 A . . .
<i>R. rhipicephali</i> (AF123719)	G				
<i>R. sibirica</i> (AF123722)	G . . . C	. . T			
<i>R. slovaca</i> (AF123723)	G . . . C		 A . . .		
<i>R. typhi</i> (L04661)	C	. . A A .	 A . . .	T	A . . T . A A . .
HYRU 176 (FSP) OmpB					
HYTR 248 (FSP) OmpB					
RHSI T47 (GP) OmpB					
RHSI T50 (GP) OmpB					
RHSI T57 (GP) OmpB					
RHSI T58 (GP) OmpB					

	810	820	830	840	850
<i>R. aeschlimannii</i> (AF123705)	A T A C G T G C T G	C T A A C C A A G A	T T A T A T A A T A	A C A C G T A C T A	A C A A T G C A G A
<i>R. africana</i> (AF123706)					
<i>R. akari</i> (AF123707)	 A . . .		 G . G . . .		 C . . .
<i>R. australis</i> (AF123709)	. . G A . . .				 G . . . C
<i>R. conorii</i> (AF123721)					
<i>R. conorii</i> (AF123726)					
<i>R. felis</i> (AF210695)					 C . . .
<i>R. helvetica</i> (AF123725)		 T . . .	 G . G . . .	 C . . .	 G . . . C
<i>R. honei</i> (AF123724)					
<i>R. japonica</i> (AF123713)				 G . . .	
<i>R. massiliae</i> (AF123714)					
<i>R. montanensis</i> (AF123716)					 T . . .
<i>R. parkeri</i> (AF123717)					
<i>R. prowazekii</i> (AF123718)	 A . T . . .	 G . . .	 G . . T . .		 G . . T . T T
<i>R. rickettsii</i> (X16353)					
<i>R. rhipicephali</i> (AF123719)					
<i>R. sibirica</i> (AF123722)					
<i>R. slovaca</i> (AF123723)					
<i>R. typhi</i> (L04661)	 A . . .	. . G . . T . . G . .	 G . G T . .		 G . . T . T T
HYRU 176 (FSP) OmpB					
HYTR 248 (FSP) OmpB					
RHSI T47 (GP) OmpB			 G . . .		
RHSI T50 (GP) OmpB			 G . . .		 T . . .
RHSI T57 (GP) OmpB					
RHSI T58 (GP) OmpB			 G . . .		

	860	870	880	890	900
<i>R. aeschlimannii</i> (AF123705)	A A A C G T A G T T	A C T A A C G A T A	T C G C A A A T A G	T T C G T T T G G A	G G T G C A C C A G
<i>R. africana</i> (AF123706)					
<i>R. akari</i> (AF123707)					
<i>R. australis</i> (AF123709)					
<i>R. conorii</i> (AF123721)					
<i>R. conorii</i> (AF123726)					
<i>R. felis</i> (AF210695)					
<i>R. helvetica</i> (AF123725)			 G . . .		
<i>R. honei</i> (AF123724)					
<i>R. japonica</i> (AF123713)	 A . . .				
<i>R. massiliae</i> (AF123714)					
<i>R. montanensis</i> (AF123716)			 G . . .		 T G . .
<i>R. parkeri</i> (AF123717)					
<i>R. prowazekii</i> (AF123718)		 A . C A . C . G	 T . G . . .	 G . A A . . C .	 A A . . . T .
<i>R. rickettsii</i> (X16353)					
<i>R. rhipicephali</i> (AF123719)					
<i>R. sibirica</i> (AF123722)					
<i>R. slovaca</i> (AF123723)					
<i>R. typhi</i> (L04661)	 T . . .	 A . C A . C . G	 A . G . . .	 G . A A . . C .	 A A . . . T .
HYRU 176 (FSP) OmpB					
HYTR 248 (FSP) OmpB					
RHSI T47 (GP) OmpB					
RHSI T50 (GP) OmpB					
RHSI T57 (GP) OmpB					
RHSI T58 (GP) OmpB					

	910	920	930	940	950
<i>R. aeschlimannii</i> (AF123705)	G T G T A G G T C A	G A A C G T T A C A	A C A T T T G T A A	A T G C A A C T A A	T A C T G C A G C A
<i>R. africana</i> (AF123706)					
<i>R. akari</i> (AF123707)					C . G . .
<i>R. australis</i> (AF123709)					C . T G . G
<i>R. conorii</i> (AF123721)					
<i>R. conorii</i> (AF123726)					
<i>R. felis</i> (AF210695)				T	. G . G
<i>R. helvetica</i> (AF123725)					C
<i>R. honei</i> (AF123724)					
<i>R. japonica</i> (AF123713)					
<i>R. massiliae</i> (AF123714)					
<i>R. montanensis</i> (AF123716)					
<i>R. parkeri</i> (AF123717)					
<i>R. prowazekii</i> (AF123718)	. . . A . . .	. . . A . T . T	. G . G C T . . G	. A T . . A . .	. . . A . . . T
<i>R. rickettsii</i> (X16353)	. . . A . . .				
<i>R. rhipicephali</i> (AF123719)					. C
<i>R. sibirica</i> (AF123722)					
<i>R. slovaca</i> (AF123723)					
<i>R. typhi</i> (L04661)	. . . C A . . .	A . . T A . . G . T	. T . . G C T . . G	. A T G	. . . A . . . T
HYRU 176 (FSP) OmpB					
HYTR 248 (FSP) OmpB					
RHSI T47 (GP) OmpB					
RHSI T50 (GP) OmpB					
RHSI T57 (GP) OmpB					
RHSI T58 (GP) OmpB					
	960	970	980	990	1000
<i>R. aeschlimannii</i> (AF123705)	T A T A A T A A T C	T T C T T T T A G C	T A A A A A T A G T	G C T G A T T C T G	C T A A C T T T G T
<i>R. africana</i> (AF123706)				A . G . .	
<i>R. akari</i> (AF123707)					G
<i>R. australis</i> (AF123709)			G		
<i>R. conorii</i> (AF123721)				A	
<i>R. conorii</i> (AF123726)				A	
<i>R. felis</i> (AF210695)					
<i>R. helvetica</i> (AF123725)					
<i>R. honei</i> (AF123724)				A . G . .	
<i>R. japonica</i> (AF123713)					T
<i>R. massiliae</i> (AF123714)					T
<i>R. montanensis</i> (AF123716)					
<i>R. parkeri</i> (AF123717)				A . G . .	
<i>R. prowazekii</i> (AF123718)	 A . G .	 G . C C .	T G T . .	. A . C A	
<i>R. rickettsii</i> (X16353)				A	
<i>R. rhipicephali</i> (AF123719)					T
<i>R. sibirica</i> (AF123722)				A . G . .	T
<i>R. slovaca</i> (AF123723)				A	
<i>R. typhi</i> (L04661)	 A . G .	 G . T C .	T G T C .	. A . C A . . A .	
HYRU 176 (FSP) OmpB					
HYTR 248 (FSP) OmpB					
RHSI T47 (GP) OmpB				A . G . .	T
RHSI T50 (GP) OmpB					
RHSI T57 (GP) OmpB					T
RHSI T58 (GP) OmpB					T
	1010	1020	1030	1040	1050
<i>R. aeschlimannii</i> (AF123705)	C G G A G C T A T C	G T T A C C G A T A	C A A G T G C G T C	C G T A A C T A A T	G C A C A A T T A G
<i>R. africana</i> (AF123706)	T		G	A	
<i>R. akari</i> (AF123707)	T T A C . . .		A G . . .	T	
<i>R. australis</i> (AF123709)	T T A C . . .		A G . . .	T	
<i>R. conorii</i> (AF123721)	T		G	A	
<i>R. conorii</i> (AF123726)	T		G	A	
<i>R. felis</i> (AF210695)	T T A C . . .		A G . . .	A	
<i>R. helvetica</i> (AF123725)	. . . A . . . T A C . . .		A G . . .		
<i>R. honei</i> (AF123724)	T		G	A	
<i>R. japonica</i> (AF123713)	. . . A		G	A	
<i>R. massiliae</i> (AF123714)			G	T	
<i>R. montanensis</i> (AF123716)	. . . T		G		T
<i>R. parkeri</i> (AF123717)	T		G	A	
<i>R. prowazekii</i> (AF123718)	A T . C . . . A .		G	T C A .	T . A . C . . A
<i>R. rickettsii</i> (X16353)	T		G	A	T
<i>R. rhipicephali</i> (AF123719)			G		
<i>R. sibirica</i> (AF123722)	T		G	A	
<i>R. slovaca</i> (AF123723)	T		G	A	
<i>R. typhi</i> (L04661)	A T . C . . . A .		G T G .	T G . . C A .	T . A . C . . A
HYRU 176 (FSP) OmpB					
HYTR 248 (FSP) OmpB					
RHSI T47 (GP) OmpB			G	T	
RHSI T50 (GP) OmpB	T		G	A	
RHSI T57 (GP) OmpB			G	T	
RHSI T58 (GP) OmpB			G	T	

	1060	1070	1080	1090	1100
<i>R. aeschlimannii</i> (AF123705)	A T G T A G C T A A	A G A T A T C C A A	G C T C A A C T T G	G T A A C A G A T T	A G G T G C T C T T
<i>R. africae</i> (AF123706)			C		
<i>R. akari</i> (AF123707)				T	A G
<i>R. australis</i> (AF123709)					
<i>R. conorii</i> (AF123721)					
<i>R. conorii</i> (AF123726)					
<i>R. felis</i> (AF210695)	A	C			
<i>R. helvetica</i> (AF123725)	A			A	G
<i>R. honei</i> (AF123724)					
<i>R. japonica</i> (AF123713)					
<i>R. massiliae</i> (AF123714)				A	
<i>R. montanensis</i> (AF123716)		A			
<i>R. parkeri</i> (AF123717)					
<i>R. prowazekii</i> (AF123718)	A T A A C	A A A C T	A A T A	T G C	A A
<i>R. rickettsii</i> (X16353)	T				
<i>R. rhipicephali</i> (AF123719)					
<i>R. sibirica</i> (AF123722)		G	A		
<i>R. slovacica</i> (AF123723)	G				
<i>R. typhi</i> (U04661)	A T A A C	A A A C T	A A T A	T G C	A
HYRU 176 (FSP) OmpB					
HYTR 248 (FSP) OmpB					
RHSI T47 (GP) OmpB					A
RHSI T50 (GP) OmpB					A
RHSI T57 (GP) OmpB					A
RHSI T58 (GP) OmpB					A
	1110	1120	1130	1140	1150
<i>R. aeschlimannii</i> (AF123705)	A G A T A T T T A G	G T A C T C C T G A	A A C T G C T G A A	A T G G C T G G A C	C T G A A G C T G G
<i>R. africae</i> (AF123706)					
<i>R. akari</i> (AF123707)		A			
<i>R. australis</i> (AF123709)					G
<i>R. conorii</i> (AF123721)					
<i>R. conorii</i> (AF123726)					
<i>R. felis</i> (AF210695)					
<i>R. helvetica</i> (AF123725)				A	G
<i>R. honei</i> (AF123724)					
<i>R. japonica</i> (AF123713)				T	T
<i>R. massiliae</i> (AF123714)					
<i>R. montanensis</i> (AF123716)					
<i>R. parkeri</i> (AF123717)					
<i>R. prowazekii</i> (AF123718)	C A	A G	T T	G T	C A A
<i>R. rickettsii</i> (X16353)					
<i>R. rhipicephali</i> (AF123719)					
<i>R. sibirica</i> (AF123722)					
<i>R. slovacica</i> (AF123723)					
<i>R. typhi</i> (U04661)	C A	A T	T	G T G	A A
HYRU 176 (FSP) OmpB					
HYTR 248 (FSP) OmpB					
RHSI T47 (GP) OmpB			C		
RHSI T50 (GP) OmpB					A
RHSI T57 (GP) OmpB			C		
RHSI T58 (GP) OmpB			C		
	1160	1170	1180	1190	1200
<i>R. aeschlimannii</i> (AF123705)	A G T A A T A	C C G G C T G T G G	T T G C T G C A G G	T G A C G A G G C T	G T T G A T A A T G
<i>R. africae</i> (AF123706)	C	C			
<i>R. akari</i> (AF123707)		T	C		C
<i>R. australis</i> (AF123709)	C	C		A	C
<i>R. conorii</i> (AF123721)	C	C			
<i>R. conorii</i> (AF123726)	C	C			
<i>R. felis</i> (AF210695)	C	C		A	C
<i>R. helvetica</i> (AF123725)	C G	C		T A	C
<i>R. honei</i> (AF123724)	C	C			
<i>R. japonica</i> (AF123713)	C	C			
<i>R. massiliae</i> (AF123714)	C	C			
<i>R. montanensis</i> (AF123716)	C	C			
<i>R. parkeri</i> (AF123717)	C	C			
<i>R. prowazekii</i> (AF123718)	T C G G	T	T	A G	A
<i>R. rickettsii</i> (X16353)	C	T			A
<i>R. rhipicephali</i> (AF123719)	C	C			G
<i>R. sibirica</i> (AF123722)	C	C			
<i>R. slovacica</i> (AF123723)	C	C			
<i>R. typhi</i> (U04661)	T C G	T	T	A G	A C A
HYRU 176 (FSP) OmpB					
HYTR 248 (FSP) OmpB					
RHSI T47 (GP) OmpB	C G C A A	C			
RHSI T50 (GP) OmpB	T C	C A			
RHSI T57 (GP) OmpB	C G C A A	C			
RHSI T58 (GP) OmpB	C G C A A	C			

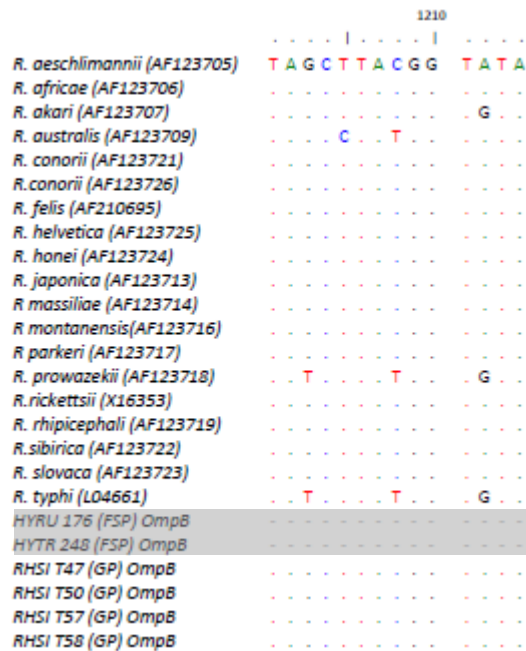
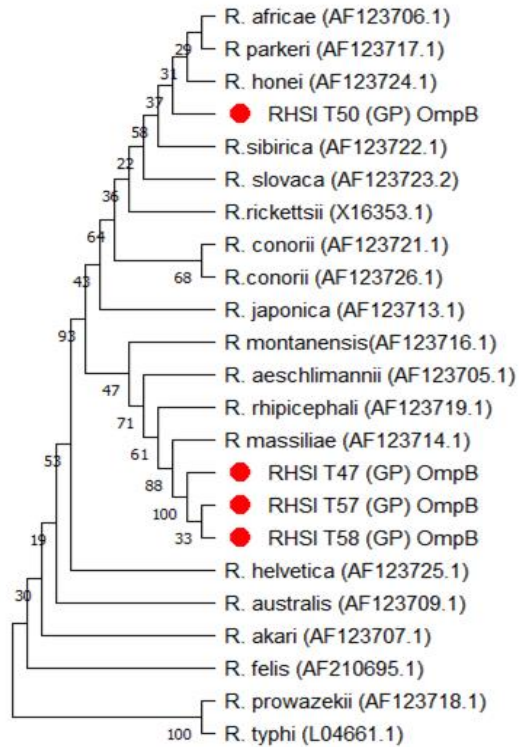


Figure 6.12 Sequence data obtained from the amplification of *Rickettsia* OmpB fragments identified in four (4) *Rh. simus* ticks (i.e. denoted by the RHSI prefixes), two (2) *H. rufipes* ticks (i.e. denoted by the HYRU prefix) aligned with the *Rickettsia* species as previously published (Fournier et al., 2003). A total of 1,214 bp positions were aligned.

A limitation of the sequence alignment analysis is the limited sample size of sequences compared. Although there are a large number of rickettsial species identified to date, the genetic sequence data for all genes for each of the species identified to date may not be readily available. It therefore becomes extremely complicated to conduct definitive comparisons – herein lies the problem with *Rickettsia* taxonomy. In our study, the large difference in product sizes and locations amplified on the *OmpB* gene meant that isolates detected in the *Rh. simus* ticks could not be compared with the *Hyalomma* ticks and were therefore placed on separate trees (see Figures 6.13 and 6.14).

Tree E: Maximum Likelihood method



Tree F: Neighbor-Joining method

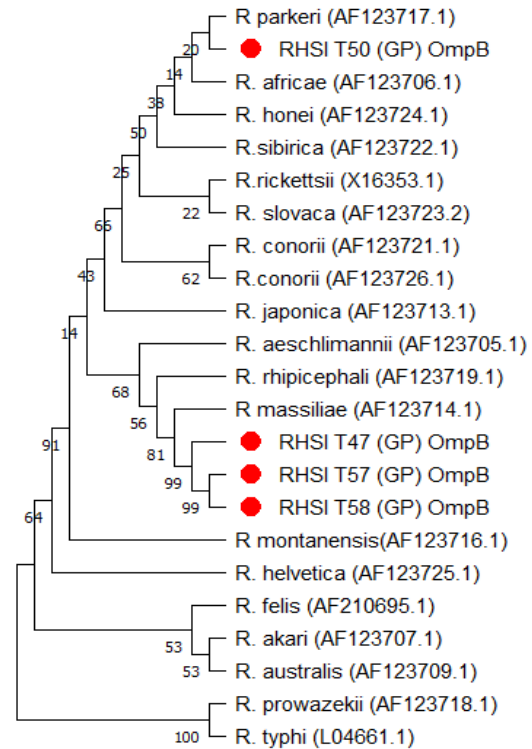
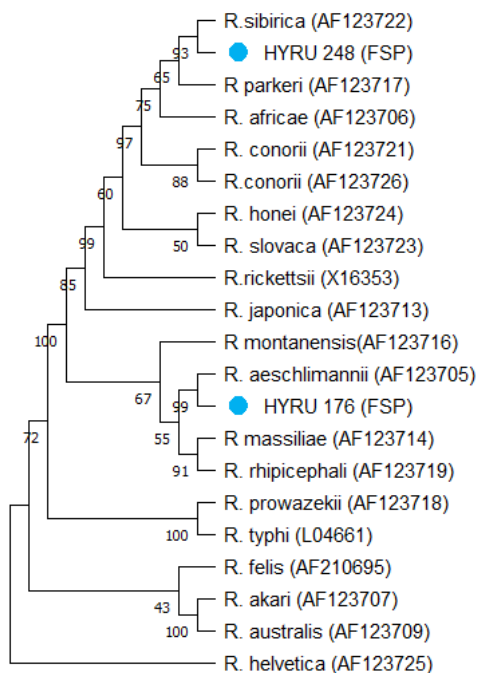


Figure 6.13 Phylogenetic analysis of *Rickettsial* Outer Membrane Protein B (OmpB) fragments detected in four *Rh. simus* (RHSI) ticks collected from Gauteng. Tree E illustrates evolutionary analysis by Maximum Likelihood method and Tree F infers evolutionary history using the Neighbor-Joining method.

Tree G: Maximum Likelihood method



Tree H: Neighbor-Joining

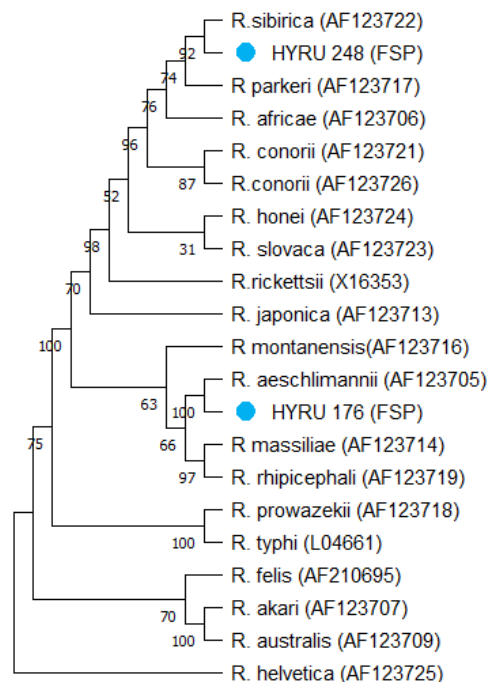


Figure 6.14 Phylogenetic analysis of *Rickettsial* Outer Membrane Protein B (OmpB) fragments detected in two (*HYRU* – *H. rufipes*) ticks collected from Free State Province. Tree G illustrates evolutionary analysis by Maximum Likelihood method and Tree H infers evolutionary history using the Neighbor-Joining method.

In Figure 6.13 – Tree E, the evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-1394.87) is shown and the percentage of trees in which the associated taxa clustered together is shown below the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. This analysis involved 23 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding and a total of 383 positions were in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

The evolutionary history was inferred using the Neighbor-Joining method in Figure 6.13 – Tree F (Saitou and Nei, 1987). The optimal tree is shown with the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown below the branches (Felsenstein, 1985b). The evolutionary distances were computed using the Tamura-Nei method (Tamura and Nei, 1993) and are in the units of the number of base substitutions per site. This analysis involved 23 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 346 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

The Maximum Likelihood method and Tamura-Nei model was used to infer evolutionary history (Tamura and Nei, 1993). The highest log likelihood (-3220.33) is shown in Figure 6.14 – Tree G and the percentage of trees in which the associated taxa clustered together is shown below the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. Twenty-one (21) nucleotide sequences and 1st+2nd+3rd+Noncoding codon positions were included in the analysis. A total of 709 positions are accounted for in the final dataset.

The Neighbor-Joining method infers evolutionary history as shown in the optimal Figure 6.14 – Tree H (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown below the branches (Felsenstein, 1985b) and the evolutionary distances were computed using the Tamura-Nei method (Tamura and Nei, 1993) and are in the units of the number of base substitutions per site. This analysis involved 21 nucleotide sequences, inclusive of 1st+2nd+3rd+Noncoding codon positions. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 679 positions in the final dataset as conducted by the MEGA11 (Tamura et al., 2021) software program.

Table 6.7 Summary of *GltA*, *OmpA* and *OmpB* sequencing results per specimen

	<i>GltA</i>		<i>OmpA</i>		<i>OmpB</i>	
	M-L method	N-J method	M-L method	N-J method	M-L method	N-J method
RHSI T47 (GP)	<i>R. rhipicephali</i>	<i>R. rhipicephali</i>	<i>R. massiliae</i>	<i>R. massiliae</i>	<i>R. massiliae</i>	<i>R. massiliae</i>
RHSI T50 (GP)	<i>R. slovaca</i>	<i>R. conorii</i>	<i>R. australis</i> / <i>R. montana</i>	<i>R. australis</i> / <i>R. montana</i>	unresolved	<i>R. parkeri</i>
RHSI T55 (GP)	<i>R. rhipicephali</i>	<i>R. rhipicephali</i>	<i>R. massiliae</i>	<i>R. massiliae</i>	No amplicon	No amplicon
RHSI T57 (GP)	<i>R. rhipicephali</i>	<i>R. rhipicephali</i>	<i>R. massiliae</i>	<i>R. massiliae</i>	<i>R. massiliae</i>	<i>R. massiliae</i>
RHSI T58 (GP)	<i>R. rhipicephali</i>	<i>R. rhipicephali</i>	<i>R. massiliae</i>	<i>R. massiliae</i>	<i>R. massiliae</i>	<i>R. massiliae</i>
HYRU 176 (FSP)	unresolved	unresolved	<i>R. aeschlimannii</i>	<i>R. aeschlimannii</i>	<i>R. aeschlimannii</i>	<i>R. aeschlimannii</i>
HYRU 177 (FSP)	unresolved	unresolved	<i>R. sibirica</i>	<i>R. sibirica</i>	No amplicon	No amplicon
HYRU 241 (FSP)	<i>R. parkeri</i>	<i>R. sibirica</i>	No amplicon	No amplicon	No amplicon	No amplicon
HYRU 242 (FSP)	<i>R. sibirica</i>	<i>R. africae</i>	No amplicon	No amplicon	No amplicon	No amplicon
HYRU 247 (FSP)	<i>R. massiliae</i>	unresolved	<i>R. aeschlimannii</i>	<i>R. aeschlimannii</i>	No amplicon	No amplicon
HYRU 248 (FSP)	unresolved	unresolved	<i>R. sibirica</i>	<i>R. sibirica</i>	<i>R. sibirica</i>	<i>R. sibirica</i>
HYRU 349 (FSP)	unresolved	unresolved	<i>R. aeschlimannii</i>	<i>R. aeschlimannii</i>	No amplicon	No amplicon
HYRU 358 (FSP)	unresolved	unresolved	<i>R. aeschlimannii</i>	<i>R. aeschlimannii</i>	No amplicon	No amplicon

*M-L: maximum likelihood method; N-J: Neighbor-joining method; RHSI: *Rhipicephalus simus*; HYRU: *Hyalomma rufipes*; GP: Gauteng Province; FSP: Free State Province.

Table 6.7 provides a summary of the tick-derived rickettsias comparing the phylogenetic trees obtained for each, based on the *gltA*, *OmpA* and *OmpB* genes. This comparison demonstrates the complexity of rickettsial phylogenetic analysis. With 96 species currently known, both validly and non-validly published (see Table 1.1), the analysis becomes extremely difficult as the required data for each species is not equally and readily available for all. In addition, the different genes have demonstrated various clusterings for example RHSI T47 (GP) clustered with *R. rhipicephali* for the

gltA and *R. massiliae* for the *OmpA* and *OmpB*. In other instances, the maximum likelihood and neighbor-joining trees cluster our rickettsias with different species as observed with RHSI T50 (GP) for *gltA* and *OmpB*, HYRU 241 (FSP) and HYRU 242 (FSP) for their *gltA* regions (*OmpA* and *OmpB* were not amplified), and HYRU 247 (FSP) for the *gltA* region. It is decidedly difficult to determine whether our rickettsias are one species or another, or whether they are entirely novel species.

6.3.3 Molecular confirmatory PCRs for Tick identifications

6.3.3.1 *Rhipicephalus* molecular confirmatory sequencing

It was attempted to confirm *Rhipicephalus* morphological tick identifications through sequencing of the ITS2 and the 18S regions as described in section 6.2.5.2. This however proved to be more complex than was initially anticipated. A 576 bp portion of the 18S region was amplified for four (4) of the 5 *Rickettsia*-positive *Rhipicephalus* ticks morphologically identified as *Rh. simus* (i.e. RHSI T47, RHSI T50, RHSI T55 and RHSI T57) and for one (1) tick that was morphologically identified as *Rh. evertsi* (*Rickettsia*-negative). The sequences obtained were then aligned against the South African voucher species (see Table 6.5) published on Genbank. Based on the alignments produced, it was demonstrated that the 18S region is fairly well conserved for all the species compared. With the exception of three (3) nucleotide variations at Po 176 for RHSI T50 and two (2) at Po's 289 and 360 for RHSI T47, there was objectively no difference between the *Rhipicephalus* species compared. It is therefore reasoned that the 18S ribosomal region in *Rhipicephalus* ticks may not be the ideal region to assess when attempting to confirm a species identification (see Figure 6.15).

The ITS-2 region was also attempted using published primers as described by Latrofa et al in 2013 (Latrofa et al., 2013) and Abdullah et al in 2016 (Abdullah et al., 2016) (see Table 6.3). On paper, these primers were objectively ideal as demonstrated in Appendix 6.3 – ITS2 primer binding positions for *Rhipicephalus* species of the supplementary material. The primers would theoretically produce a 1,202 bp product, however in practice, attempts to amplify and sequence the region proved taxing and unsuccessful. Internal primers were also designed in-house to aid with the amplification of smaller products, however these attempts were also unsuccessful.

Furthermore, due to the extremely limited volumes of DNA available for all assays, the material was used up and not available for retesting.

As a result of failed attempts to confirm the morphological identifications through molecular techniques, it was decided to defer to the morphological identifications made by expert acarologist.

	10	20	30	40	50
<i>Rh. appendiculatus</i> (KY457501)	A T T C T A G A G C	T A A T A C A T G C	A G T G A G C C T G	G A G C C C T T T G	G G T A A C G G G T
<i>Rh. appendiculatus</i> (KY457500)					
<i>Rh. evertsi</i> (KY457503)					
<i>Rh. evertsi</i> (KY457502.)					
<i>Rh. maculatus</i> (KY457505)					
<i>Rh. maculatus</i> (KY457504)					
<i>Rh. microplus</i> (KY457506)					
<i>Rh. simus</i> (KY457508)					
<i>Rh. zambeziensis</i> (KY457509)					
<i>Rh. zambeziensis</i> (KY457507)					
RHEV T41 - 18S					
RHSI T47 - 18S					
RHSI T50 - 18S					
RHSI T55 - 18S					
RHSI T57 - 18S					
	60	70	80	90	100
<i>Rh. appendiculatus</i> (KY457501)	G C T T T T A T T A	G A C C A A G A T C	G A T C G G G T T T	C G G C C C G T A T	T G T G T G G T G A
<i>Rh. appendiculatus</i> (KY457500)					
<i>Rh. evertsi</i> (KY457503)					
<i>Rh. evertsi</i> (KY457502.)					
<i>Rh. maculatus</i> (KY457505)					
<i>Rh. maculatus</i> (KY457504)					
<i>Rh. microplus</i> (KY457506)					
<i>Rh. simus</i> (KY457508)					
<i>Rh. zambeziensis</i> (KY457509)					
<i>Rh. zambeziensis</i> (KY457507)					
RHEV T41 - 18S					
RHSI T47 - 18S					
RHSI T50 - 18S					
RHSI T55 - 18S					
RHSI T57 - 18S					
	110	120	130	140	150
<i>Rh. appendiculatus</i> (KY457501)	C T C T G G A T A A	C T T T G T G C T G	A T C G C A T G G C	C A C G A G C C G G	C G A C G T T T C T
<i>Rh. appendiculatus</i> (KY457500)					
<i>Rh. evertsi</i> (KY457503)					
<i>Rh. evertsi</i> (KY457502.)					
<i>Rh. maculatus</i> (KY457505)					
<i>Rh. maculatus</i> (KY457504)					
<i>Rh. microplus</i> (KY457506)					
<i>Rh. simus</i> (KY457508)					
<i>Rh. zambeziensis</i> (KY457509)					
<i>Rh. zambeziensis</i> (KY457507)					
RHEV T41 - 18S					
RHSI T47 - 18S					
RHSI T50 - 18S					
RHSI T55 - 18S					
RHSI T57 - 18S					
	160	170	180	190	200
<i>Rh. appendiculatus</i> (KY457501)	T T C A A G T G T C	T G C C T T A T C A	A C T T T C G A T G	G T A G G T T A C T	T G C T T A C C A T
<i>Rh. appendiculatus</i> (KY457500)					
<i>Rh. evertsi</i> (KY457503)					
<i>Rh. evertsi</i> (KY457502.)					
<i>Rh. maculatus</i> (KY457505)					
<i>Rh. maculatus</i> (KY457504)					
<i>Rh. microplus</i> (KY457506)					
<i>Rh. simus</i> (KY457508)					
<i>Rh. zambeziensis</i> (KY457509)					
<i>Rh. zambeziensis</i> (KY457507)					
RHEV T41 - 18S					
RHSI T47 - 18S					
RHSI T50 - 18S			G		
RHSI T55 - 18S					
RHSI T57 - 18S					

	210	220	230	240	250
<i>Rh. appendiculatus</i> (KY457501)	G G T T G T T A C G	G G T A A C G G A G	A A T C A G G G T T	C G A T T C C G G A	G A G G G A G C C T
<i>Rh. appendiculatus</i> (KY457500)					
<i>Rh. evertsi</i> (KY457503)					
<i>Rh. evertsi</i> (KY457502.)					
<i>Rh. maculatus</i> (KY457505)					
<i>Rh. maculatus</i> (KY457504)					
<i>Rh. microplus</i> (KY457506)					
<i>Rh. simus</i> (KY457508)					
<i>Rh. zambeziensis</i> (KY457509)					
<i>Rh. zambeziensis</i> (KY457507)					
RHEV T41 - 18S					
RHSI T47 - 18S					
RHSI T50 - 18S					
RHSI T55 - 18S					
RHSI T57 - 18S					
	260	270	280	290	300
<i>Rh. appendiculatus</i> (KY457501)	G A G A A A C G G C	T A C C A C A T C C	A A G G A A G G C A	G C A G G C G C G C	A A A T T A C C C A
<i>Rh. appendiculatus</i> (KY457500)					
<i>Rh. evertsi</i> (KY457503)					
<i>Rh. evertsi</i> (KY457502.)					
<i>Rh. maculatus</i> (KY457505)					
<i>Rh. maculatus</i> (KY457504)					
<i>Rh. microplus</i> (KY457506)					
<i>Rh. simus</i> (KY457508)					
<i>Rh. zambeziensis</i> (KY457509)					
<i>Rh. zambeziensis</i> (KY457507)					
RHEV T41 - 18S					
RHSI T47 - 18S					
RHSI T50 - 18S					
RHSI T55 - 18S					
RHSI T57 - 18S					
	310	320	330	340	350
<i>Rh. appendiculatus</i> (KY457501)	C T C C C G G C A C	G G G G A G G T A G	T G A C G A A A A A	T A A C A A T A C G	G G A C T C T T T T
<i>Rh. appendiculatus</i> (KY457500)					
<i>Rh. evertsi</i> (KY457503)					
<i>Rh. evertsi</i> (KY457502.)					
<i>Rh. maculatus</i> (KY457505)					
<i>Rh. maculatus</i> (KY457504)					
<i>Rh. microplus</i> (KY457506)					
<i>Rh. simus</i> (KY457508)					
<i>Rh. zambeziensis</i> (KY457509)					
<i>Rh. zambeziensis</i> (KY457507)					
RHEV T41 - 18S					
RHSI T47 - 18S					
RHSI T50 - 18S					
RHSI T55 - 18S					
RHSI T57 - 18S					
	360	370	380	390	400
<i>Rh. appendiculatus</i> (KY457501)	G A G G C C C G T	A A T T G A A A T G	A G T A C A C T C T	A A A T C C T T T A	A C G A G G A T C A
<i>Rh. appendiculatus</i> (KY457500)					
<i>Rh. evertsi</i> (KY457503)					
<i>Rh. evertsi</i> (KY457502.)					
<i>Rh. maculatus</i> (KY457505)					
<i>Rh. maculatus</i> (KY457504)					
<i>Rh. microplus</i> (KY457506)					
<i>Rh. simus</i> (KY457508)					
<i>Rh. zambeziensis</i> (KY457509)					
<i>Rh. zambeziensis</i> (KY457507)					
RHEV T41 - 18S					
RHSI T47 - 18S					
RHSI T50 - 18S					
RHSI T55 - 18S					
RHSI T57 - 18S					

6.3.3.2 *Hyalomma* molecular confirmations using the COI mitochondrial region

The *Hyalomma* COI region proved to be more useful for confirming morphological identifications. A product of 426 was amplified and compared with the closest species identified via GenBank BLAST application. Due to the limited genomic data available for *H. rufipes* and *H. truncatum* in South Africa, a comparison was not possible, hence the comparison with the closest hits on BLAST. Both Maximum Likelihood phylogenetic tree (Figure 6.16) and nucleotide alignments (Figure 6.17) presented confirm the morphological identifications as *H. rufipes* ticks.

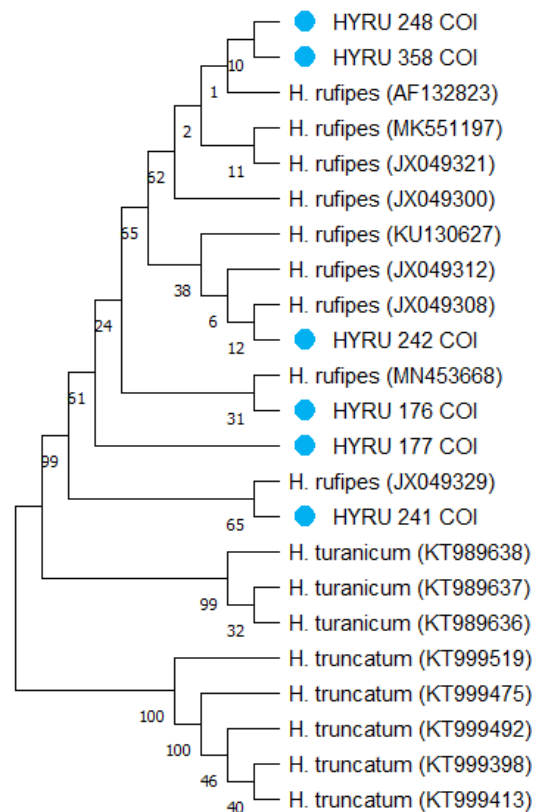


Figure 6.16 The evolutionary history of five (5) *H. rufipes* compared with closest BLAST matches, was inferred by using the Maximum Likelihood method and Tamura-Nei model.

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-1006.81) is shown. The percentage of trees in which the associated taxa clustered together is shown below the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. This analysis involved 23 nucleotide

sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 416 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

	10	20	30	40	50
<i>H. rufipes</i> (KU130627)	A A C A T T A A T T	G G T A A T G A T C	A A A T T T A T A A	T G T A A T T G T A	A C A G C C C A T G
<i>H. rufipes</i> (JX049312)					
<i>H. rufipes</i> (JX049308)					
<i>H. rufipes</i> (MN453668)					
<i>H. rufipes</i> (AF132823)					
<i>H. rufipes</i> (JX049329)					
<i>H. rufipes</i> (MK551197)					
<i>H. rufipes</i> (JX049321)					
<i>H. rufipes</i> (JX049300)					
<i>H. turanicum</i> (KT989638)	G				 C ..
<i>H. turanicum</i> (KT989637)	G				 C ..
<i>H. turanicum</i> (KT989636)	G				 C ..
<i>H. truncatum</i> (KT999398)	G G			 T	 G
<i>H. truncatum</i> (KT999413)	G G			 T	 G
<i>H. truncatum</i> (KT999492)	G G			 T	 G
<i>H. truncatum</i> (KT999475)	G G			 T	 G
<i>H. truncatum</i> (KT999519)	T C	 C		 C .. T ..	
HYRU 176 COI					
HYRU 177 COI					
HYRU 241 COI					
HYRU 242 COI					
HYRU 248 COI					
HYRU 358 COI					
	60	70	80	90	100
<i>H. rufipes</i> (KU130627)	C T T T T G T T A T	A A T T T T T T T T	A T A G T T A T A C	C T A T T A T A A T	T G G A G G A T T T
<i>H. rufipes</i> (JX049312)					
<i>H. rufipes</i> (JX049308)					
<i>H. rufipes</i> (MN453668)					
<i>H. rufipes</i> (AF132823)					
<i>H. rufipes</i> (JX049329)					
<i>H. rufipes</i> (MK551197)					
<i>H. rufipes</i> (JX049321)					
<i>H. rufipes</i> (JX049300)					
<i>H. turanicum</i> (KT989638)					
<i>H. turanicum</i> (KT989637)					
<i>H. turanicum</i> (KT989636)					
<i>H. truncatum</i> (KT999398)	 C	 C			 G
<i>H. truncatum</i> (KT999413)	 C	 C			 G
<i>H. truncatum</i> (KT999492)	 C	 C			 G
<i>H. truncatum</i> (KT999475)	 C	 C			 G
<i>H. truncatum</i> (KT999519)	 C				 G C
HYRU 176 COI					
HYRU 177 COI					
HYRU 241 COI					
HYRU 242 COI					
HYRU 248 COI					
HYRU 358 COI					
	110	120	130	140	150
<i>H. rufipes</i> (KU130627)	G G T A A T T G G T	T A G T C C C A A T	C A T A T T A G G A	T C C C C A G A C A	T A G C A T T T C C
<i>H. rufipes</i> (JX049312)					
<i>H. rufipes</i> (JX049308)					
<i>H. rufipes</i> (MN453668)					
<i>H. rufipes</i> (AF132823)					
<i>H. rufipes</i> (JX049329)					
<i>H. rufipes</i> (MK551197)					
<i>H. rufipes</i> (JX049321)					
<i>H. rufipes</i> (JX049300)					
<i>H. turanicum</i> (KT989638)	 A			 T T ..	
<i>H. turanicum</i> (KT989637)	 A			 T T ..	
<i>H. turanicum</i> (KT989636)	 A			 T T ..	
<i>H. truncatum</i> (KT999398)	 A T	 T		 T T ..	 G C ..
<i>H. truncatum</i> (KT999413)	 A T	 T		 T T ..	 G C ..
<i>H. truncatum</i> (KT999492)	 A T	 T		 T T ..	 G C ..
<i>H. truncatum</i> (KT999475)	 A T	 T		 T T ..	 G
<i>H. truncatum</i> (KT999519)	 A A		 C	 T	
HYRU 176 COI					
HYRU 177 COI					
HYRU 241 COI					
HYRU 242 COI					
HYRU 248 COI					
HYRU 358 COI					

	160	170	180	190	200
<i>H. rufipes</i> (KU130627)	T C G A A T A A A T	A A T A T A A G C T	T T T G A T T A T T	A C C T C C T T C T	C T T T T T T T A C
<i>H. rufipes</i> (JX049312)					
<i>H. rufipes</i> (JX049308)					
<i>H. rufipes</i> (MN453668)					
<i>H. rufipes</i> (AF132823)				C	
<i>H. rufipes</i> (JX049329)					
<i>H. rufipes</i> (MK551197)				C	
<i>H. rufipes</i> (JX049321)				C	
<i>H. rufipes</i> (JX049300)				C	
<i>H. turanicum</i> (KT989638)					
<i>H. turanicum</i> (KT989637)					
<i>H. turanicum</i> (KT989636)					
<i>H. truncatum</i> (KT999398)	T	A	C	A	T A C G T
<i>H. truncatum</i> (KT999413)	T	A	C	A	T A C G T
<i>H. truncatum</i> (KT999492)	T	A	C	A	T A C G T
<i>H. truncatum</i> (KT999475)	T	A	C	A	T A C G T
<i>H. truncatum</i> (KT999519)	C	A	C	A	A C G T
HYRU 176 COI					
HYRU 177 COI					
HYRU 241 COI					
HYRU 242 COI					
HYRU 248 COI				C	
HYRU 358 COI				C	
	210	220	230	240	250
<i>H. rufipes</i> (KU130627)	T A T T A A A T T C	A T C T T T A A T T	G A A T C G G G A G	C A G G A A C T G G	A T G A A C A G T T
<i>H. rufipes</i> (JX049312)					
<i>H. rufipes</i> (JX049308)					
<i>H. rufipes</i> (MN453668)					
<i>H. rufipes</i> (AF132823)					
<i>H. rufipes</i> (JX049329)					
<i>H. rufipes</i> (MK551197)					
<i>H. rufipes</i> (JX049321)					
<i>H. rufipes</i> (JX049300)					
<i>H. turanicum</i> (KT989638)					
<i>H. turanicum</i> (KT989637)					
<i>H. turanicum</i> (KT989636)					
<i>H. truncatum</i> (KT999398)	G C	A C T	A	A	
<i>H. truncatum</i> (KT999413)	G	A C T	A	A	
<i>H. truncatum</i> (KT999492)	G	A C T	A	A	
<i>H. truncatum</i> (KT999475)	G	A C T	A	A	
<i>H. truncatum</i> (KT999519)		C	G A	A	
HYRU 176 COI					
HYRU 177 COI	G				
HYRU 241 COI					
HYRU 242 COI					
HYRU 248 COI					C
HYRU 358 COI					
	260	270	280	290	300
<i>H. rufipes</i> (KU130627)	T A T C C T C C A C	T T T C T T C A A A	T T T A T C T C A T	T A C G G A C C T T	C G G T T G A C T T
<i>H. rufipes</i> (JX049312)					
<i>H. rufipes</i> (JX049308)					
<i>H. rufipes</i> (MN453668)					
<i>H. rufipes</i> (AF132823)					
<i>H. rufipes</i> (JX049329)					
<i>H. rufipes</i> (MK551197)					
<i>H. rufipes</i> (JX049321)					
<i>H. rufipes</i> (JX049300)					
<i>H. turanicum</i> (KT989638)	C				
<i>H. turanicum</i> (KT989637)	C				
<i>H. turanicum</i> (KT989636)	C				
<i>H. truncatum</i> (KT999398)	C	C	C	T	A
<i>H. truncatum</i> (KT999413)	C	C	C	T	A
<i>H. truncatum</i> (KT999492)	C	C	C	T	A
<i>H. truncatum</i> (KT999475)	C	C	C	T	A
<i>H. truncatum</i> (KT999519)	C		C	T	A
HYRU 176 COI					
HYRU 177 COI					
HYRU 241 COI					
HYRU 242 COI					
HYRU 248 COI					
HYRU 358 COI					

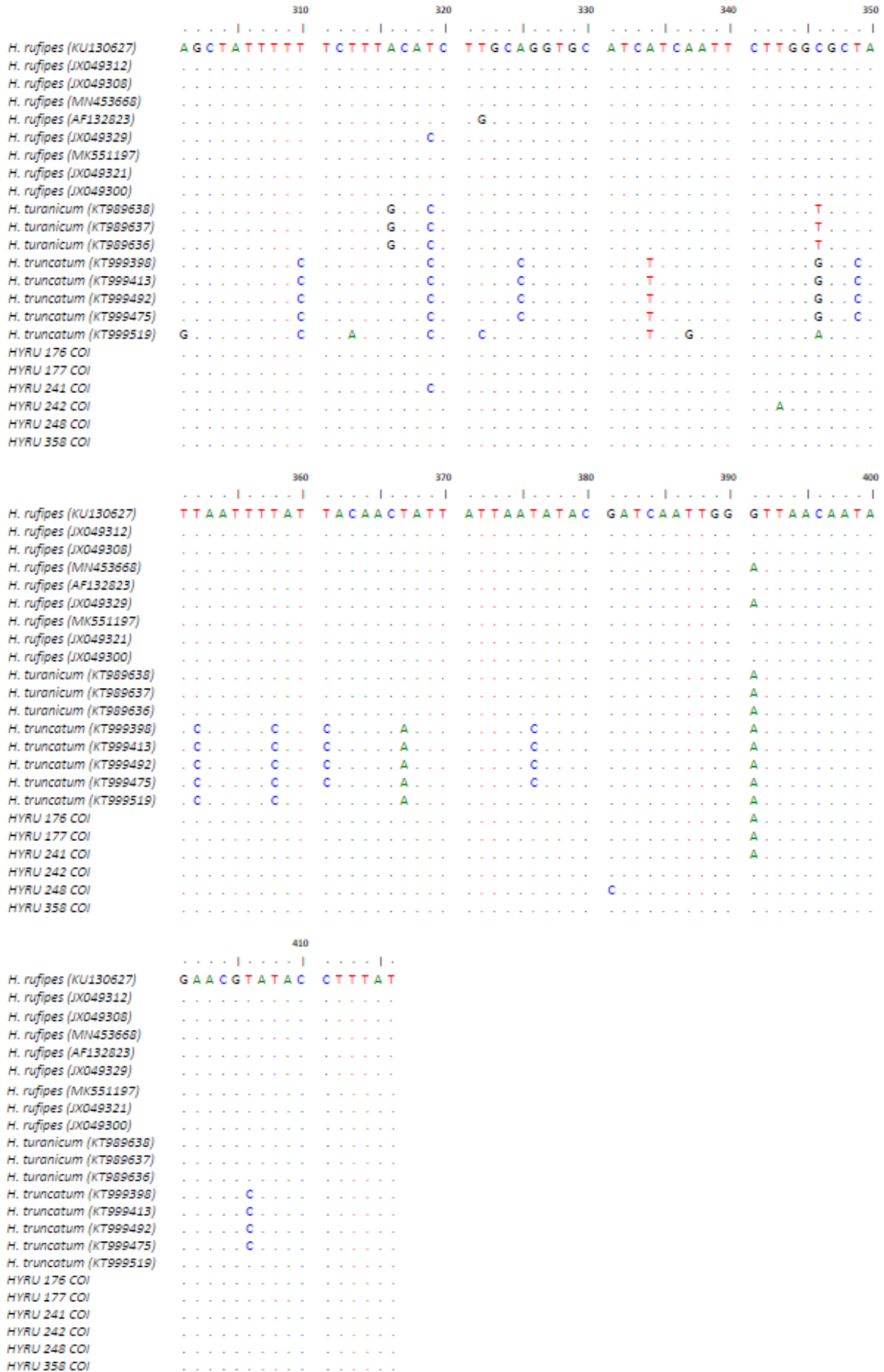


Figure 6.17 *Hyalomma* sequence alignments confirming morphological identifications.

6.4 DISCUSSION

Rickettsia species phylogeny is controversial (Igolkina et al., 2023) with the conventional criteria used for the characterisation of bacterial species (i.e. 16S rRNA gene sequence similarity, genomic G+C content and DDH relatedness) do not apply to rickettsial species (Diop et al., 2020). The current criteria for the description of a novel *Rickettsia* species is based on a multiphasic investigative approach focused on MLST results, phylogenetic classification and phenotypic data descriptions – most notably its human pathogenic potential, geographic distribution and relationship with vector arthropods (Diop et al., 2020). Complying with the aforementioned criteria, however, still does not mean that the species will be a validly published species until type strains are stored by two (2) official culture collections in separate countries, and by publication in the International Journal of Systematic and Evolutionary Microbiology validation list (Diop et al., 2020).

Since its inception in 2003, MLST targeted towards the investigation of five (5) genes (i.e. 16S rRNA, *gltA*, *ompA*, *ompB* and *sca4*) has aided rickettsial classification and taxonomy of isolates (Diop et al., 2020). It is currently the foundation for the characterization of rickettsial species and was primary technique allowing for the definition of sequence-based species boundaries appropriate for all *Rickettsia* species (Diop et al., 2020). A multi-gene sequencing technique with gene sequence-based conditions for rickettsial isolate identification to genus, group and species levels, was proposed (Fournier and Raoult, 2007) with the acknowledgement that the method lacked the discriminatory power to differentiate one species from another (Fournier and Raoult, 2007). Phylogenic investigations targeting divergent genes such as the *gltA*, the 17-kDa protein-encoding gene and autotransporter *sca* family including: *ompA*, *ompB*, *sca4*, *sca1*, and *sca2* have been shown to be useful (Fournier and Raoult, 2009). The *gltA* gene generally assists in providing good resolution for rickettsias outside the SFG, but identifies four (4) statistically-supported clusters within the SFG, namely: *R. rickettsii* cluster containing within it *R. conorii*, *R. peacockii*, *R. honei*, *R. rickettsii*, *R. africae*, *R. parkeri*, *R. sibirica*, *R. slovaca*, *R. heilongjiangensis*, and *R. japonica*; the *R. massiliae* cluster consisting of *R. massiliae*, *R. rhipicephali*, *R. aeschlimannii*, *R. raoultii*, and *R. montanensis*; the *R. akari* cluster consisting of *R. akari*, *R. felis*, and *R. australis*; and the *R. helvetica* cluster, containing *R. helvetica*, *R. asiatica*, and *R. tamurae* (Fournier and Raoult,

2009). Based on these clusterings, it can be surmised that our identified rickettsias are classified, based on *gltA* sequences, in the SFG clusters as follows: RHSI T50, HYRU 177, HYRU 241, HYRU 242 and HYRU 248 in the *R. rickettsii* cluster; and RHSI T47, RHSI T55, RHSI T57, RHSI T58, HYRU 176, HYRU 247, and HYRU 358 in the *R. massiliae* cluster.

Based on the MLST criteria presented by Fournier and Raoult' publication (Fournier and Raoult, 2009) elucidating "Polyphasic taxonomic guidelines for classification of rickettsiae", our bacterial *gltA* region will have needed to be at least 86.5% similar to at least one (1) validated *Rickettsia* species. Having met that requirement with all our rickettsias sufficiently similar (>99% similarity) to various rickettsial species in the *gltA* region (see Table 6.8), the second step would be to determine whether our *Rickettsia* species were new species. In order to satisfy these criteria, more than one (1) gene would need to be sequenced and be sufficiently similar with validated *Rickettsia* species (i.e. *rrs* > 99.8%, *gltA* > 99.9%, *OmpA* > 98.8%, *OmpB* > 99.2%, and *sca4* > 99.3%) (Fournier and Raoult, 2009). In our study, we attempted to sequence *OmpA* and *OmpB* regions for each of our isolates, with variable rates of success, with some of our isolates failing to yield products for one or both of the additional genes. Nevertheless, we sequenced the products that were obtained and determined that only two (2) of our isolates produced concordant results where *gltA* and *OmpA* and *OmpB* provided demonstrated sufficient similarity to validated species to be identified as the same, i.e. HYRU 177 and HYRU 248 as *R. sibirica* (potentially subsp. *mongolitimonae*) – a species that has previously been reported in South Africa (Ndip et al., 2011) and considered a human pathogen as its first identification was in a patient afflicted with lymphangitis, headache, and fever (Essbauer et al., 2018, Pretorius and Birtles, 2004, Parola, 2006).

The remainder of our rickettsias pose a conundrum as the *gltA*, *OmpA* and *OmpB* sequences did not appear to resolve the species. It is however likely, based on a recent publication of a South African study looking at rodents and their ectoparasites for the harborage of rickettsias (Essbauer et al., 2018), that RHSI T47, RHSI T55, RHSI T57, and RHSI T58 are in fact examples of 'Candidatus' *R. muridii* (KT835137) and that RHSI T50 is a 'Candidatus' *R. rhabdomydis* (KT835131) (Essbauer et al., 2018). The remainder of our isolates (i.e. HYRU 176, HYRU 241, HYRU 242, HYRU

247, HYRU 349 and HYRU 358) will require additional work to determine whether they are novel *Rickettsia* species. It is suspected that HYRU 176, HYRU 247, and HYRU 358 are members of the same species with their *gltA* gene being > 99.3% similar to *R. conorii* subsp. *raoultii* (MT178338) and the *OmpA* 100% similar to *R. aeschlimannii* (MK922658). Although the *OmpB* did not amplify for HYRU 247, and HYRU 358, the gene was amplified and sequenced for HYRU 176 with 100% similarity to *R. aeschlimannii* (MK215218). It is also plausible that HYRU 241 and HYRU 349 are examples of the same species as both are significantly similar to *R. parkeri* (MK814825) according to their *gltA* gene. This however cannot be corroborated with certainty as there is only one (1) genetic region to compare since the *OmpA* only for HYRU 241 and not for HYRU 349, and *OmpB* for neither of the two (2) isolates. Finally, HYRU 242 seemed to be a different species to the other isolates as only the *gltA* gene was successfully amplified and sequenced. When the sequence similarity was sought on Genbank its *gltA* demonstrated 100% similarity to *R. africae* (MN025497). Further work is required to ascertain whether HYRU 242 is truly a *R. africae* or a different species that would be revealed through sequencing of additional genes as is the case for our other isolates.

There are a vast number of rickettsial species identified in the last two (2) decades both validly and non-validly published. Our summary table (Table 1.1) presented the complete list, as it currently stands, on the 'List of Prokaryotic names with Standing in Nomenclature' (LPSN) website accessed in July 2023 (<https://lpsn.dsmz.de/search?word=rickettsia>). Counting species with misspelled names with the corrected names as one (1) species, a total of 96 *Rickettsia* species appear on the list, both validly and non-validly published. It is also known that the list continues to grow and currently excludes a large number of unclassified isolates or genotypes that await classification and even some recently- identified and published 'candidatus' species, two (2) of which we encountered in our study, namely 'Candidatus' *R. muridii* and that RHSI T50 is a 'Candidatus' *R. rhabdomydis* (Essbauer et al., 2018a). It is apparent that the complexity of rickettsial taxonomy may have to do with several genetic and evolutionary features within these relatively small (1.1–2.3 Mbp) bacterial genome (El Karkouri et al., 2022). For example, rickettsias have demonstrated a significant amount of inter-species genomic synteny, reductive evolution potentially due to a strictly intracellular existence, a high A + T content, an

absence of some metabolic pathways that lead to the dependence on host cells to provide the absent metabolites, and inconstant dissemination of plasmids (El Karkouri et al., 2022).

Table 6.8 *Rickettsial species sequence similarities in as per BLAST alignments*

	GltA		OmpA		OmpB	
	Species	Sequence similarity	Species	Sequence similarity	Species	Sequence similarity
RHSI T47 (GP)	<i>R. rhipicephali</i> (MN477897) and <i>R. massiliae</i> (KY640405)	100 %	'Candidatus' <i>R. muridii</i> isolate Suedafrika0033 (KT835137)	100 %	<i>R. massiliae</i> (OL634840.1)	96.43 %
RHSI T50 (GP)	'Candidatus' <i>R. barbariae</i> (MZ351056)	100 %	'Candidatus' <i>R. rhabdomydis</i> isolate Suedafrika0012 (KT835131.1)	100 %	<i>Rickettsia</i> sp. strain Ga-Seema (MG953290)	100 %
RHSI T55 (GP)	<i>R. rhipicephali</i> (MN477897) and <i>R. massiliae</i> (KY640405)	100 %	'Candidatus' <i>R. muridii</i> isolate Suedafrika0033 (KT835137)	99.78 %	-	-
RHSI T57 (GP)	<i>R. rhipicephali</i> (MN477897) and <i>R. massiliae</i> (KY640405)	100 %	'Candidatus' <i>R. muridii</i> isolate Suedafrika0033 (KT835137)	100 %	<i>R. massiliae</i> (OL634840)	96.34 %
RHSI T58 (GP)	<i>R. rhipicephali</i> (MN477897) and <i>R. massiliae</i> (KY640405)	100 %	'Candidatus' <i>R. muridii</i> isolate Suedafrika0033 (KT835137)	100 %	<i>R. massiliae</i> (KT861886)	99.12 %
HYRU 176 (FSP)	<i>R. conorii</i> subsp. <i>raoultii</i> (MT178338)	99.4 %	<i>R. aeschlimannii</i> (MK922658)	100 %	<i>R. aeschlimannii</i> (MK215218)	100 %
HYRU 177 (FSP)	<i>R. sibirica</i> (KX421823)	99.4 %	<i>R. sibirica</i> subsp. <i>Mongolitimonae</i> (MK922654)	100 %	-	-
HYRU 241 (FSP)	<i>R. parkeri</i> (MK814825), <i>R. conorii</i> subsp. <i>conorii</i> (MN399865) and <i>R. africae</i> (MH938655)	97.5 %	-	-	-	-
HYRU 242 (FSP)	<i>R. africae</i> (MN025497)	100 %	-	-	-	-
HYRU 247 (FSP)	<i>R. conorii</i> subsp. <i>raoultii</i> (MT178338)	100 %	<i>R. aeschlimannii</i> (MK922658)	100 %	-	-
HYRU 248 (FSP)	<i>R. sibirica</i> (KX421823)	99.1 %	<i>R. sibirica</i> subsp. <i>Mongolitimonae</i> (MK922654)	100 %	<i>R. sibirica</i> (KX686602)	100 %
HYRU 349 (FSP)	<i>R. parkeri</i> (MK814825)	99.5 %	<i>R. aeschlimannii</i> (MK922658)	100 %	-	-
HYRU 358 (FSP)	<i>R. conorii</i> subsp. <i>raoultii</i> (MT178338)	99.38 %	<i>R. aeschlimannii</i> (MK922658)	100 %	-	-

6.5 CONCLUSION

Our study is a true demonstration of the complexity of rickettsial phylogenetic analysis. With 96 species currently known, both validly and non-validly published (see Table 1.1), the analysis becomes extremely difficult as the required genetic data for each species are not equally and readily available for all species. Though literature has shown that various of genes are targeted for phylogenetic and taxonomic studies, no one gene target is more effective than another (Blanton, 2019). It is especially difficult to determine species based on limited product sizes, as was the case for our study that had 200 bp products for our *gltA* region. One cannot help but think if the picture would be different if longer products were available for analysis. In the last 20-years, significant improvements in DNA sequencing techniques have permitted access to complete genomic sequences, all inside a short timeframe and at an affordable cost providing unparalleled access to vast amounts of sequence data (Diop et al., 2020). It is anticipated that taxonomic classification of prokaryotes will continue to rapidly evolve, rendering conventional techniques obsolete (Diop et al., 2020).

CHAPTER 7 – CONCLUDING SUMMARY

Rickettsiosis is considered as an emerging group of diseases of growing public health concern in many locations around the globe. In recent years the knowledge of the diversity and geographical distribution of Rickettsial species have evolved at an accelerated pace primarily due to the advent of molecular typing technologies. In South Africa, tick bite fever is a very common occurrence mostly diagnosed and managed based on clinical features only. The knowledge of rickettsial diversity and the causative agents of tick bite fever in South Africa is understudied. In this study, we attempt to address questions related to laboratory diagnostic capacity for tick bite fever in the country, characterize the causative agents of severe cases of tick bite fever and explore the diversity of rickettsiae from different tick species in different locations in the country.

In this study we sought to better understand the human rickettsiosis, and diagnostic capabilities for detection of rickettsial diseases, and to assess the diversity of *Rickettsia* species in South Africa. To achieve its aims, the study was conducted in two (2) parts. Part one (1) of the study sought to address the first aim which was to evaluate commercial assays and the diagnostic testing capabilities available for *Rickettsia* in South Africa. The commercially-available serological test kits on offer for the detection of rickettsioses in South Africa, were obtained and evaluated against a panel of residual clinical samples. Ultimately, two (2) ELISA and two (2) IFA kits were compared with each other with the expectation that the results obtained using one (1) assay would be the same or similar with another. The results of this analysis were presented in Chapter 3, and surprisingly the null hypothesis was rejected, due to the significant differences found between all the kits. Granted, limitations of this investigation were: 1) the lack of availability of fully characterized sera to use in the evaluation; 2) the unavailability of baseline population titers for endemic regions necessary for accurate setting of the cut off values; 3) limited sample number; 4) potentially compromised sample quality due to repetitive freeze-thawing since samples used were residual from previous studies; and 5) single operator testing for most of the assay experiments. This study proved valuable in that it highlighted the

deficiencies of commercial diagnostic offerings and additional work necessary to better equip laboratories and the medical fraternity as a whole to identify and diagnose rickettsioses. Commercially available kits are extremely useful, but require some in-house evaluation, validation and possibly adaptation that would assist diagnostic laboratories in better serving their populations. Often commercial assays are used as “plug and play” solutions where simply following manufacturer protocols yield a result with the accuracy of said assay-derived result never coming into question. It is therefore recommended that further epidemiological work be conducted to assist laboratories in setting assay cut-off values for endemic populations. It is also important that laboratories have access to fully characterized samples for in-house validation purposes. This can be achieved through subscription to external quality assurance programs (EQAPs). Unfortunately, these programs are limited in South Africa with the only offerings being those subscribed to from distant shores and endemic conditions. A way to circumvent this limitation is through the establishment of within country ILCs.

To further investigate the diagnostic testing capabilities within the country, an inter-laboratory comparison (ILC) was conducted to investigate the performance and capacity gaps of laboratories in South Africa offering routine serological tests for *Rickettsia* diagnostics. This objective was discussed in Chapter 4, and has been published (Trataris-Rebisz et al., 2019). Briefly, the ILC consisting of a standardised panel of specimens was shared with three (3) private laboratories that routinely offer *Rickettsia* diagnostic assays. Laboratories were to treat the specimens in a manner consistent with their routine procedures and to submit their results for comparative analyses. Although the overall agreement between participating laboratories for IgG assays was good, result interpretations were aligned with commercial kit provider recommendations and not dependent on endemic epidemiological cut-off adjustments. In addition, the IgM results were less encouraging, with one (1) of the participating laboratories reporting false-positive results for half of the panel. This leads to the concerns regarding patient management since clinicians may rely on laboratory confirmations to guide treatment regimes, especially when confronted with an array of non-specific symptoms and non-response to treatments provided. The implications of unreliable laboratory diagnostic results may lead to dire consequences for patient care. It is however acknowledged that this comparison served as a once-off “snap shot” assessment and therefore could not track improvements over time. Furthermore, the assessment was targeted towards

serological techniques, but with the global trend toward molecular methodologies, particularly for serologically problematic infections such as rickettsioses, serological techniques are being replaced with molecular ones. One could speculate that the participant laboratories would have provided more concordant results if molecular methods were used, although this cannot be proven or disproved without further studies being conducted. That been said, serological assays are still heavily relied upon for rickettsial diagnosis, as was presented in the questionnaire portion of the ILC. It is acknowledged, that although IFA is considered the reference method for rickettsial diagnostics, the assay requires highly skilled and experienced operators, and specialized equipment and conditions for any valuable assertions to be made. This is probably due to the subjective nature of the assay since a visualized instance could appear positive to one operator and negative to another. In addition, IFA slides experience a degradation of fluorescence over time and exposure to the light source thereby affecting how the results are read. It is therefore emphasized that ILCs (both serological and molecular-based panels) be routinely prepared and shared among South African Laboratories to aid proficiency assessment in *Rickettsia* diagnostics. It is also recommended that routine, passive surveillance programs be set up for known 'hot spot' *Rickettsia* areas to monitor the baseline immunity in the endemic populations. As mentioned previously, these epidemiological data would greatly facilitate to optimization criteria (e.g. cutoff values and baseline titers) for current diagnostic assays in the country.

Part 2 of this study was directed towards achieving a better understanding of the actual rickettsial species causing severe patient disease outcomes and the species being harbored by several tick species often encountered in the human-animal-arthropod interface where rickettsial transmission is likely to occur. In doing so, the second and third aims presented at the commencement of the study, would be satisfied; specifically, to characterize the *Rickettsia* associated with severe cases of TBF in South Africa, and to investigate the presence of *Rickettsia* species in ectoparasitic vectors collected from different sites around South Africa. In Chapter 5, molecular sequencing was performed to type the Rickettsial species involved in severe cases of TBF in a case series of patients presenting with suspected CCHF but diagnosed with TBF between 2011-2018. Clinical aspects and genetic characterization of the

implicated *Rickettsia* species were presented in an attempt to better understand severe outcomes for patients afflicted with TBF. Tick bite fever was demonstrated in this study to be an essential differential diagnosis for CCHF. Both TBF and CCHF share geographic distribution, primary vectors and symptom presentations and the same has been reported elsewhere (de Almeida et al., 2010; Blumenthal et al., 2021). In South Africa, rickettsial infection is generally attributed to either *R. africae* or *R. conorii* with the latter generally associated with the more severe clinical presentation and potentially fatal outcomes (Blumenthal et al., 2021). Our *gltA* sequence results corroborate the notion that *R. conorii* is likely the causative agent of severe clinical presentation, although it cannot be definitively confirmed since we had a very limited supply of residual patient material for further molecular characterisation and phylogenetic analysis looking at *OmpA* and *OmpB* genes. Despite the limitations of this study, it is emphasized that clinicians need to consider rickettsial infections for patients presenting with severe yet, non-specific symptomologies, if the patient is to receive the correct treatment in time. It is acknowledged that a limitation of this study is that the sequences obtained could not be compared with other *gltA* sequences from South Africa, since primers were designed 'in-house' and amplified a different region on the *gltA* gene. It is recommended that additional work be conducted in the study of severe outcome TBF. It is also highlighted that clinicians become sensitized to the prospect of severe and fatal patient outcomes in what is usually considered a benign and self-limiting disease in South Africa. In cases that are suspected of being CCHF/VHFs, TBF should also be investigated as a possible aetiology for the disease. Clinicians are encouraged to submit eschar swabs and/or eschar biopsies, where available, for PCR analysis in addition to the blood samples submitted, as the likelihood of detecting *Rickettsia*, if present, is improved.

To address the final aim, ticks collected from different sites in South Africa were screened for the presence and genetic diversity of *Rickettsia* species. Chapter 6 yielded some interesting findings despite the small number of *Rickettsia*-positive ticks detected. *Rhipicephalus* ticks were found to be harboring two (2) recently-identified species in South Africa 'Candidatus' *R. muridii* and 'Candidatus' *R. rhabdomydis*. The *H. rufipes* ticks demonstrated a larger *Rickettsia* species diversity with *Hylomma* ticks found to be carrying *R. sibirica* subsp. *mongolitimonae*, and possibly two (2) to three

(3) currently uncharacterized species in the remaining six (6) *Hyalomma* ticks. A limitation of the study was that due to the small size of the ticks, the entire tick was used up in the extraction process and therefore extractions and/or repeat PCR investigations could not be conducted. This meant that for some of the *OmpA* and *OmpB* regions, no sequence was obtained. Tick identifications using the *ITS2* gene as the target for the confirmation of *Rhipicephalus* tick morphological identifications could not successfully be run for the *Rickettsia*-positive ticks. In addition, there are relatively few South African tick sequences available for comparisons to be made particularly for tick identifications. It is recommended that further studies be conducted looking at improving tick molecular identifications. Through the progress achieved using next generation whole genome sequencing, it is anticipated that both *Rickettsia* taxonomy, tick identification and the interaction at the various interfaces will propel the study of *Rickettsia* species forward and perhaps render conventional techniques obsolete. Until that day, may this study lead to many others.

In conclusion, there remains a fair amount to work necessary to be done to improve *Rickettsia* diagnostics in South Africa. This can be achieved through the establishment of regular ILC or quality assurance programs set up within country to continuously challenge laboratories to ensure their methodologies are providing the most accurate results possible. Laboratories are encouraged to perform in-house optimization of assays taking into account population-specific epidemiological data. Passive surveillance data is necessary to assist in determining this population-specific epidemiological data. It is also encouraged that more molecular typing assays be set up in laboratories to better understand the causative species for rickettsioses in South Africa. Finally, biosurveillance programs targeting ticks and their pathogens remain vitally important as early warning sentinel surveillance. In order to anticipate human new and emerging diseases, we need to know what is happening in the vectors we encounter.

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SUPPLEMENTARY MATERIALS

Chapter 3 Appendices

Appendix 3.1 - *Rickettsia* diagnosis: advantages and disadvantages of serological techniques.

Table 3.1 Serological assay methodologies adapted to *Rickettsia* diagnosis and the advantages and disadvantages of each technique.

Type of Assay	Principle	Advantages	Disadvantages
Complement fixation (CF)	Detects the presence of specific antibodies in the patient's serum by using complement, a biologically labile serum factor that causes the immune cytolysis i.e. lysis of antibody coated cells. The patient serum sample is first added to the known antigens (in this instance rickettsial antigens). If specific antibodies to a particular antigen are present in the serum, antigen-antibody complexes form. When complement is then added, it will react with the complexes and become depleted. When sheep red blood cells (sRBCs) are then added, the complement will be used up and cannot react with the sRBCs. If no specific antibodies are present, the complement will not be depleted and will lyse the sRBCs turning the solution pink.	• Provides results comparable with MIF (Eremeeva et al., 1994) • Relatively easy to perform with minimal demand for equipment and reagents. • Adaptable test to many types of antigens, soluble or particulate • Reading is easy (lysis, no lysis) • Economical (relatively cheap to perform)	• Relies on the presence of antibodies that may not be present in early infection. • This is a largely outdated test that has been replaced with newer serological methodologies such as ELISA. • Not sensitive - cannot be used for immunity screening • Time consuming and labor intensive • Often non-specific e.g. cross-reactivity • Some of components need to be fresh (RBC's, Complement) • Anti-complementary activity (Péter et al., 1987) • Results vary according to antigen production method and antigen amount used in the assay
Enzyme-linked immunosorbent assays (ELISA)	ELISAs are immunoassays often used for the quantification of the levels of a specific target within a sample (e.g. serum or plasma, etc.). These assays are usually run in 96-well microplates coated with a particular antigen. Patient sera are diluted and applied to the coated wells. Microplates are then incubated to allow for the serum antibodies to bind to the antigens. Wells are then washed to remove unreacted serum proteins, and a peroxidase-labelled anti-human conjugate is added to label the bound antibody. Following further incubation and a second wash-step aimed at removing unbound conjugate, a substrate is added to quantify the bound peroxidase portion of the conjugate. The blue colour-development is directly proportional to the amount of reactive	• High sensitivity and specificity • High throughput: normally available in a 96-well plate format, but can be easily adapted to 384-well plates. • Easy to perform: easy to follow protocols with little hands-on time. • Quantitative • Possibility to test various sample types: serum, plasma, cellular and tissue extracts, urine, and saliva among others. • Relatively cheap to perform. • Adaptable to various antigens. • More standardized than IFAs – results are reproducible and objectively read.	• Temporary readouts: detection is based on enzyme/substrate reactions and therefore readout must be obtained in a short time span. • Limited information: information limited to amount or presence in a sample. • Require specialized readers to measure the absorbance. • Due to delayed antibody responses usually associated with <i>Rickettsia</i> infection, seroconversion must be demonstrable – therefore acute and convalescent samples tested together must demonstrate a fourfold or greater rise in titre in order to confirm a current or recent infection.

	serum antibody. This timed reaction is then interrupted with a stop solution that changes the colour from blue to yellow and stabilizes the final colour intensity. This colour intensity (Absorbance) is then measured at a wavelength of 450nm on a micro-titre plate reader or spectrophotometer.		
Immunofluorescence assays (IFA)	Detects anti-rickettsial antibodies that bind to fixed antigens (e.g., <i>Rickettsia conorii</i>) on a slide and visualized by a fluorescein-labelled anti-species-specific (e.g., human or dog) G or M immunoglobulin. The timing of sample collection is a key factor to consider as the detection of antibodies depends on the time from onset of illness. For the confirmation of a recent or current infection seroconversion must be demonstrable, four-fold or greater rise in antibody titre, between acute and convalescent samples (Maggi et al., 2014).	• The persistence of circulating antibodies can be advantageous since antibodies may be detectable during chronic intravascular infections, when a pathogen may be circulating below the limit detectable by PCR or isolated in tissues not routinely tested by molecular techniques (Maggi et al., 2014). • This test is relatively easy to perform and other than the Fluorescent microscope, requires very little additional equipment, i.e. incubator, timer and pipette. • To perform an IFA is not time consuming and a result is available in 2-3 hours. • Relatively sensitive and specific depending on the kit being used. • The sensitivity of IFA 2 weeks after the onset of illness is 94% to 100% • Can be set up to test for multiple antigens simultaneously on a single well on a slide	• Lack of detectable antibodies in first 7–10 days of illness, or longer (>25 days) for <i>R. africae</i> means that this test may not work in the first few days after onset. Presumptive clinical diagnosis should be confirmed by testing 2 sequential serum samples taken 2–6 weeks apart. • Extensive serologic cross-reactivity within <i>Rickettsia</i> groups (SFG, TG), so a +ve titre only indicates exposure to a rickettsial species as well as cross-reactivity related other pathogen infections. • False-positive IgM antibodies (McQuiston et al., 2014). • Reading is subjective (i.e. what may appear positive to one operator may appear negative to another) and skill operators are therefore required to read the slides. • Reading the slides is often complicated by background fluorescence. • Fluorescence fades so slides need to be stored/handled in the dark and viewed quickly upon completion of the test. • A negative does not rule out infection as there are several factors to be considered when reading the results. • IFA methods have an exceptionally low negative predictive value (Paddock, 2013, Biggs et al., 2016)
Latex agglutination (LA)	Serological techniques used to detect certain antigens or antibodies in a serum or plasma sample of a patient suspected of having a <i>Rickettsia</i> infection. The sample homogenised with latex beads coated with the specific antigen	• Easy to perform and read – visual clumping observed • Cheap • Does not require specialized equipment or highly skilled personnel. • Results are quickly obtained – rapid test.	• Provides insufficient evidence to confirm a new SFR illness and use hampers full understanding of SFR epidemiology and the incidence of disease (Binder et al., 2019) • Subject to the same limitations as many of the other serological assays e.g. delayed

	or antibody. A positive result appears as clustering of the latex particles.		antibody development preventing acute infection diagnosis in early onset of infection • Cross-reactivity between species and with other related pathogens – false positives or negatives.
Weil-Felix (WF)	Detects antigenic cross-reactivity between antibodies produced in acute rickettsial infections, with antigens of <i>OX</i> (<i>OX 19</i> , <i>OX 2</i> , and <i>OXK</i>) strains of <i>Proteus</i> species, through a process of testing dilutions of patient's serum against different <i>Proteus</i> strain suspensions (Mahajan, 2012). The test can be performed on a slide or in a test tube.	• Cheapest, easiest and most widely available test - cost effective option resource-limited settings (Cortenraad, 2014, Nimboor et al., 2018) • The antigens necessary (<i>OX2</i>, <i>OX19</i>, and <i>OXK</i>) can be obtained commercially. • Visible agglutination (slide method) or flocculation/granulation (tube method) is indicative of a positive result	• Poor sensitivity and specificity - resulting in many erroneous and unreliable results (Cortenraad, 2014, Sood et al., 2013, Nimboor et al., 2018) • Non-specific agglutination test detects anti-rickettsial antibodies in patient's serum • Imprecise assay • High rate of false negatives • Agglutinating antibodies detectable at 5 to 10 days after symptom onset – detected antibodies mainly IgM (La Scola and Raoult, 1997a, La Scola and Raoult, 1997b)
Western Blotting (WB)	WB is a useful assay for the microbiological diagnosis of rickettsioses due to it often appearing positive earlier in the course of the disease. It detects lipopolysaccharide (early-occurring antibodies) reactive antibodies. It is also useful in confirming a diagnosis as it detects late-occurring antibodies for specific protein antigens located in the rickettsial outer membrane. When WB is performed in conjunction with cross-absorbed sera, the infecting rickettsial species can be identified (Cortenraad, 2014). It is utilized to separate and identify proteins. In principle, a protein mixture is separated by gel electrophoresis based on molecular weight and type. The results are thereafter transferred to a membrane where a band produced for each protein. Following the incubation of the membrane, antibodies specific to the protein of interest are labelled. Unbound antibody is washed off allowing only the bound antibody to the protein of interest to remain. Detection of the bound antibodies is achieved by film development. Since antibodies exclusively bind to a protein of interest, a sole band should	• Western blot analysis can identify the species through cross-adsorption	• Complimentary test to the IFA, on its own may give false positives due to antibody cross-reactivity (Cortenraad, 2014). • Homologous and heterologous antibodies are not detected by Western blot (Fang et al., 2017). • Not easily performed in routine laboratories – not commercially available (Blanton, 2019).

	be visible. The band thickness correlates with the amount of protein present particularly when run against a standard (Mahmood & Yang, 2012).		
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Appendix 3.1 - Wits HREC amended ethical clearance certificate – M120667

Human Research Ethics Committee (Medical)

Research Office Secretariat: Senate House Room SH 10005, 10th floor. Tel +27 (0)11-717-1252
Medical School Secretariat: PV Tobias Building, 2nd Floor Tel +27 (0)11-717-2700
Private Bag 3, Wits 2050, www.wits.ac.za. Fax +27 (0)11-717-1265



20 October 2015

Jaqueline Weyer

NICD

1 Modderfontein Road

Sandringham

2031

Sent by email to: vanessaq@nicd.ac.za

Dear Dr Weyer

Re: Protocol Ref no: M120667

Protocol Title: Identifying Zoonotic Aetiologies in Adults with Acute Febrile Illness and Baseline Seroprevalence for Selected Zoonotic Conditions at the Human-Wildlife-Livestock Interface, Mpumalanga, SA

Principal Investigator: Dr Vanessa Quan

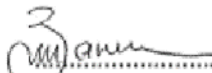
Protocol Amendment

This letter serves to confirm that the Chairman of the Human Research Ethics Committee (Medical) has approved the amended protocol on the above mentioned study, as detailed in your letter dated 11 September 2015.

- Protocol version 1.4 dated 27 April 2015

Thank you for keeping us informed and updated.

Yours Sincerely,



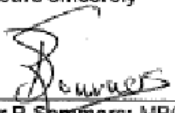
Ms Zanele Ndlovu

Administrative Officer

Human Research Ethics Committee (Medical)



Appendix 3.3 – UP HREC ethical clearance certificate – 222/2017

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance. • FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022. • IRB 0000 2295 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.		UNIVERSITEIT VAN PRETORIA UNIVERSITY OF PRETORIA YUNIBESITHI YA PRETORIA Faculty of Health Sciences Research Ethics Committee 1/06/2017						
Approval Certificate New Application								
Ethics Reference No.: 222/2017 Title: ZOONOTIC RICKETTSIOSES IN SOUTH AFRICA: DIAGNOSTIC, TAXONOMIC AND EPIDEMIOLOGICAL ASPECTS (ZORICKSA) Dear Mrs Anastasia TratarisRebisz The New Application as supported by documents specified in your cover letter dated 21/04/2017 for your research received on the 26/04/2017, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 31/05/2017. Please note the following about your ethics approval: • Ethics Approval is valid for 4 years • Please remember to use your protocol number (222/2017) on any documents or correspondence with the Research Ethics Committee regarding your research. • Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research. Ethics approval is subject to the following: • The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and • The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee. We wish you the best with your research. Yours sincerely  Dr R Sommers; MBChB; MMed (Int); MPharm, PhD Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria <i>The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).</i> <table style="width: 100%; font-size: small;"><tr><td style="width: 33%;">☎ 012 358 3084</td><td style="width: 33%;">✉ deepeka.behari@up.ac.za / fhsethics@up.ac.za</td><td style="width: 33%;">🌐 http://www.up.ac.za/healthethics</td></tr><tr><td colspan="3">📍 Private Bag X323, Arcadia, 0007 - Tswelopele Building, Level 4, Room 60, Gezins, Pretoria</td></tr></table>			☎ 012 358 3084	✉ deepeka.behari@up.ac.za / fhsethics@up.ac.za	🌐 http://www.up.ac.za/healthethics	📍 Private Bag X323, Arcadia, 0007 - Tswelopele Building, Level 4, Room 60, Gezins, Pretoria		
☎ 012 358 3084	✉ deepeka.behari@up.ac.za / fhsethics@up.ac.za	🌐 http://www.up.ac.za/healthethics						
📍 Private Bag X323, Arcadia, 0007 - Tswelopele Building, Level 4, Room 60, Gezins, Pretoria								

Chapter 4 Appendices

Appendix 4.1 - *Rickettsia* Laboratory Diagnostics Questionnaire



Centre for Emerging, Zoonotic and Parasitic Diseases
 1 Mulderfontein Road
 Sandringham, 2102
 Tel: (011) 555 0331 / 0306 | Fax: (011) 555 0447
 Contact: Anastasia Tiatans-Rebisi
 Email: anastasia@nicd.ac.za

Tick Bite Fever / *Rickettsia* Laboratory Diagnostics Questionnaire

Laboratory Name _____ Contact Person _____ Contact Number _____
 Contact email address _____
 Address _____

Please pick options from the drop down menus as it applies to your laboratory, alternatively provide information in free text:

1. General Questions		
a	Does your laboratory offer routine <i>Rickettsia</i> diagnostic testing?	
b	If no, do you refer your <i>Rickettsia</i> diagnostic test requests to another lab?	
c	If yes (c) to which laboratory do you refer your <i>Rickettsia</i> diagnostic test requests?	
d	Please provide a reason for your laboratory not offering <i>Rickettsia</i> testing?	
e	If other, please specify the reason.	
2. PCR / Molecular Testing Techniques		
a	Does your laboratory offer <i>Rickettsia</i> diagnostic testing by PCR?	
b	If yes, is it a commercially available test or in-house developed test?	
c	If commercially available, which kit/assay?	
d	If other, please specify the kit/assay used.	
e	If in-house developed, please provide a description of the in-house method.	
f	If other, please describe.	
g	If based on a published method, please provide reference.	
h	Is it a validated test?	
i	Is it an ISO accredited test?	
j	What type of PCR is it?	
k	What <i>Rickettsia</i> species are targeted?	
l	If other, please specify the target species.	
m	What region/gene is targeted?	
n	If other, please specify the target region/gene.	
o	What sample type is tested?	
p	If other, please specify the sample type.	
q	How are results interpreted?	
r	Please provide a description of the interpretation.	Negative result
		Positive result
		Indeterminate result
s	When are PCR done?	
t	If other, please specify when PCRs are done.	
3. Serological Testing Techniques		
a	Does your laboratory offer <i>Rickettsia</i> diagnostic testing by serology?	
b	If yes, is it a commercially available test or in-house developed test?	
c	If commercially available, which kit?	
d	If other, please specify the kit/assay used.	
e	Is this for antigen detection or serology (i.e. antibody detection)?	
f	Is it a validated test?	
g	Is it an accredited test?	
h	What type of serological test is it?	
i	If other, please specify the type of test used.	
j	What <i>Rickettsia</i> species are targeted?	
k	If other, please specify the target species.	
l	What immunoglobulins are targeted?	
m	If other, please specify the targeted immunoglobulins.	
n	What sample type is tested?	
o	If other, please specify the sample type.	
p	How are results interpreted?	
q	Please provide a description of the interpretation.	Negative result
		Positive result
		Indeterminate result
r	When are serological assays done?	
s	If other, please specify when serological assays are performed.	
4. Other Test Assays		
a	Does your laboratory offer the Weil-Felix antigen test for <i>Rickettsia</i> diagnosis?	
b	Does your laboratory offer any other <i>Rickettsia</i> diagnostic test not described previously?	
c	If yes please describe the test used.	
5. Quality Assurance		
a	Is your laboratory ISO accredited?	
b	Does your laboratory subscribe to an External Quality Assurance Program (EQA) for <i>rickettsial</i> tests offered?	
c	If yes, which program?	
d	If other, please specify the name of the EQA program to which you subscribe.	
e	Would your laboratory be interested in participating in an inter laboratory comparison for <i>Rickettsia</i> diagnostics with the NICD?	
f	If yes, for PCR or serological techniques?	

Thank you for your participation.

Appendix 4.2 - Participant ILC Instructions and Response Sheet



CENTRE FOR EMERGING ZOOONOTIC AND PARASITIC DISEASES

1 Modderfontein Road, Sandringham, 2192

Tel: +27 (0)11 555 0331/0306 Fax: +27 (0)11 555 0447

Reference: Rickettsia Interlaboratory Comparison 2018

Dear Participant

RE: TICK-BITE FEVER (RICKETTSIA) INTER-LABORATORY COMPARISON

Thank you for agreeing to participate in this tick-bite fever (TBF) inter-laboratory comparison program for serology. The program focuses on TBF caused by rickettsial species. Your participation is highly valued. Kindly read the instructions below and submit your responses by no later than 21st April 2018 to anastasian@nicd.ac.za.

INSTRUCTIONS

Read the scenario below and perform serological testing routinely performed in your laboratory for investigation of TBF.

The panel consists of 10 serum samples (50 µl) each.

Kindly confirm receipt of the samples in your laboratory and inform about the condition of the specimens upon receipt.

Upon receipt in your laboratory, please freeze the sera specimens at -20°C until they are to be tested as incorrect storage may affect the integrity of the specimen.

Note: All specimens are derived from human patients or volunteers and should therefore be treated as potentially infectious utilizing standard Biosafety level 2 precautions. Source materials have not been screened for Hepatitis B surface antigen, Hepatitis C antibody and HIV-1/2 (AIDS) antibodies, and therefore it cannot be assured that these or other infectious agents are not present in the samples.

SCENARIO

A group of university students attended an ecology field trip at a facility in Mpumalanga Province, South Africa. The students were conducting an environmental impact assessment for the area and were required to walk through communal grazing lands with long grass. The area is endemic to various tick species and almost all the students reported tick bites. Several of the students presented shortly thereafter with severe flu-like symptoms, including fever, headache and malaise. Few of the students reported developing an eschar at the tick bite site. .

Conduct routine serological testing for tick bite fever (IgG and IgM) on the serum samples provided.

Once all surveys have been evaluated, a consensus report will be compiled and sent out to all participants.

Physical Address: 1 Modderfontein Road, Sandringham, Johannesburg, South Africa
Chairperson: Prof Eric Buch Acting CEO: Dr Karmani Chetty
Postal Address: Private Bag X4, Sandringham, 2131, South Africa
Tel: +27 (0) 11 386 6400 Fax: +27 (0) 11 882 0596 www.nicd.ac.za
Practice number: 5200296

TICK-BITE FEVER (RICKETTSIA) INTER-LABORATORY COMPARISON

Laboratory Name			
Contact Person		Contact Number	
Contact email address			
Survey number	2018/01		
Date sent		Date received	
Date processed		Date of submission	
Condition of specimens			
Kit/Test Information: Test: for example IgM, Total antibodies(Ab) Method: for example ELISA (enzyme-linked immunosorbance assay), IFA (immunofluorescence Assay)			
Kit manufacturer/s:			
Kit Supplier/s:			
Test	Method	Lot number	Expiry Date
Sample number	Test method used	IgM result/s	IgG result/s
2018/01 – 001			
2018/01 – 002			
2018/01 – 003			
2018/01 – 004			
2018/01 – 005			
2018/01 – 006			
2018/01 – 007			
2018/01 – 008			
2018/01 – 009			
2018/01 – 010			

General questions: Please answer the following general questions relating to the test/s conducted.			
Is it a validated test?		Is it an ISO accredited test?	
Is the specificity of the assay known? (i.e. which anti-rickettsial antibodies do you expect to be able to detect?)			
Is the cross-reactivity of the assay with other bacterial antigens known, if so elaborate			
Interpretation of results: Please provide details about any comments that accompany a test result issued on official results.			
Result issued	Specific comments accompanying the result on the official report issued		
Positive			
Equivocal			
Negative			
Other comments included on the reports			
Thank you for your contribution.			

Chapter 6 Appendices

Appendix 6.1 - Section 20 approval (protocol no. 12/11/1/1/13)



agriculture, forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

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

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/4/1

Dr Jacqueline Weyer
Centre for Emerging, Zoonotic and Parasitic Diseases
National Institute for Communicable Diseases
1 Modderfontein Road
Sandringham
2192

Dear Dr Weyer

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

Your fax / memo / letter/ Email dated 3 May 2017, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

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

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. Samples may only be collected from farms in the Northern Cape, Free State and Limpopo provinces, for which provincial state veterinary permission has been granted;
4. Only a registered waste disposal company may be utilised for the removal of waste generated during the study.

Title of research/study: Viral and bacterial pathogens of public health importance associated with ticks and fleas in South Africa

Researcher (s): Dr Jacqueline Weyer

Institution: Centre for Emerging, Zoonotic and Parasitic Diseases, National Institute for Communicable Diseases

Your Ref./ Project Number: 131/11

Our Ref. Number: 12/11/1/1/13

Expiry Date: December 2020

Kind regards



DR MPHOMAJA
DIRECTOR OF ANIMAL HEALTH

Date: 2017 -09- 04

- 2 -

CLASSIFICATION: CONFIDENTIAL

SUBJECT: VIRAL AND BACTERIAL PATHOGENS OF PUBLIC HEALTH IMPORTANCE ASSOCIATED WITH TICKS AND FLEAS IN SOUTH AFRICA

Appendix 6.2 – 18S primer binding positions for *Rhipicephalus* species ticks

	10	20	30	40	50
<i>Rh. appendiculatus</i> (KY457501)	C A T T A A C A T C	A G T T A T G G T T	C C T T A G A T C G	T T T C T T C C T A	C T T G G A T A A C
<i>Rh. appendiculatus</i> (KY457500)					
<i>Rh. evertsi</i> (KY457503)					
<i>Rh. evertsi</i> (KY457502)					
<i>Rh. maculatus</i> (KY457505)					
<i>Rh. maculatus</i> (KY457504)					
<i>Rh. microplus</i> (KY457506)					
<i>Rh. simus</i> (KY457508)					
<i>Rh. zambeziensis</i> (KY457509)					
<i>Rh. zambeziensis</i> (KY457507)					
18S F					
18S R					
	60	70	80	90	100
<i>Rh. appendiculatus</i> (KY457501)	T G T G G C A A T T	C T A G A G C T A A	T A C A T G C A G T	G A G C C T G G A G	C C C T T T G G G T
<i>Rh. appendiculatus</i> (KY457500)					
<i>Rh. evertsi</i> (KY457503)					
<i>Rh. evertsi</i> (KY457502)					
<i>Rh. maculatus</i> (KY457505)					
<i>Rh. maculatus</i> (KY457504)					
<i>Rh. microplus</i> (KY457506)					
<i>Rh. simus</i> (KY457508)					
<i>Rh. zambeziensis</i> (KY457509)					
<i>Rh. zambeziensis</i> (KY457507)					
18S F					
18S R					
	110	120	130	140	150
<i>Rh. appendiculatus</i> (KY457501)	A A C G G G T G C T	T T T A T T A G A C	C A A G A T C G A T	C G G G T T T C G G	C C C G T A T T G T
<i>Rh. appendiculatus</i> (KY457500)					
<i>Rh. evertsi</i> (KY457503)					
<i>Rh. evertsi</i> (KY457502)					
<i>Rh. maculatus</i> (KY457505)					
<i>Rh. maculatus</i> (KY457504)					
<i>Rh. microplus</i> (KY457506)					
<i>Rh. simus</i> (KY457508)					
<i>Rh. zambeziensis</i> (KY457509)					
<i>Rh. zambeziensis</i> (KY457507)					
18S F					
18S R					
	160	170	180	190	200
<i>Rh. appendiculatus</i> (KY457501)	G T G G T G A C T C	T G G A T A A C T T	T G T G C T G A T C	G C A T G G C C A C	G A G C C G G C G A
<i>Rh. appendiculatus</i> (KY457500)					
<i>Rh. evertsi</i> (KY457503)					
<i>Rh. evertsi</i> (KY457502)					
<i>Rh. maculatus</i> (KY457505)					
<i>Rh. maculatus</i> (KY457504)					
<i>Rh. microplus</i> (KY457506)					
<i>Rh. simus</i> (KY457508)					
<i>Rh. zambeziensis</i> (KY457509)					
<i>Rh. zambeziensis</i> (KY457507)					
18S F					
18S R					
	210	220	230	240	250
<i>Rh. appendiculatus</i> (KY457501)	C G T T T C T T T C	A A G T G T C T G C	C T T A T C A A C T	T T C G A T G G T A	G G T T A C T T G C
<i>Rh. appendiculatus</i> (KY457500)					
<i>Rh. evertsi</i> (KY457503)					
<i>Rh. evertsi</i> (KY457502)					
<i>Rh. maculatus</i> (KY457505)					
<i>Rh. maculatus</i> (KY457504)					
<i>Rh. microplus</i> (KY457506)					
<i>Rh. simus</i> (KY457508)					
<i>Rh. zambeziensis</i> (KY457509)					
<i>Rh. zambeziensis</i> (KY457507)					

18S F	-----	-----	-----	-----	-----
18S R	-----	-----	-----	-----	-----
	260	270	280	290	300
<i>Rh. appendiculatus</i> (KY457501)	T T A C C A T G G T	T G T T A C G G G T	A A C G G A G A A T	C A G G G T T C G A	T T C C G G A G A G
<i>Rh. appendiculatus</i> (KY457500)	-----	-----	-----	-----	-----
<i>Rh. evertsi</i> (KY457503)	-----	-----	-----	-----	-----
<i>Rh. evertsi</i> (KY457502)	-----	-----	-----	-----	-----
<i>Rh. maculatus</i> (KY457505)	-----	-----	-----	-----	-----
<i>Rh. maculatus</i> (KY457504)	-----	-----	-----	-----	-----
<i>Rh. microplus</i> (KY457506)	-----	-----	-----	-----	-----
<i>Rh. simus</i> (KY457508)	-----	-----	-----	-----	-----
<i>Rh. zambeziensis</i> (KY457509)	-----	-----	-----	-----	-----
<i>Rh. zambeziensis</i> (KY457507)	-----	-----	-----	-----	-----
18S F	-----	-----	-----	-----	-----
18S R	-----	-----	-----	-----	-----
	310	320	330	340	350
<i>Rh. appendiculatus</i> (KY457501)	G G A G C C T G A G	A A A C G G C T A C	C A C A T C C A A G	G A A G G C A G C A	G G C G C G C A A A
<i>Rh. appendiculatus</i> (KY457500)	-----	-----	-----	-----	-----
<i>Rh. evertsi</i> (KY457503)	-----	-----	-----	-----	-----
<i>Rh. evertsi</i> (KY457502)	-----	-----	-----	-----	-----
<i>Rh. maculatus</i> (KY457505)	-----	-----	-----	-----	-----
<i>Rh. maculatus</i> (KY457504)	-----	-----	-----	-----	-----
<i>Rh. microplus</i> (KY457506)	-----	-----	-----	-----	-----
<i>Rh. simus</i> (KY457508)	-----	-----	-----	-----	-----
<i>Rh. zambeziensis</i> (KY457509)	-----	-----	-----	-----	-----
<i>Rh. zambeziensis</i> (KY457507)	-----	-----	-----	-----	-----
18S F	-----	-----	-----	-----	-----
18S R	-----	-----	-----	-----	-----
	360	370	380	390	400
<i>Rh. appendiculatus</i> (KY457501)	T T A C C C A C T C	C C G G C A C G G G	G A G G T A G T G A	C G A A A A A T A A	C A A T A C G G G A
<i>Rh. appendiculatus</i> (KY457500)	-----	-----	-----	-----	-----
<i>Rh. evertsi</i> (KY457503)	-----	-----	-----	-----	-----
<i>Rh. evertsi</i> (KY457502)	-----	-----	-----	-----	-----
<i>Rh. maculatus</i> (KY457505)	-----	-----	-----	-----	-----
<i>Rh. maculatus</i> (KY457504)	-----	-----	-----	-----	-----
<i>Rh. microplus</i> (KY457506)	-----	-----	-----	-----	-----
<i>Rh. simus</i> (KY457508)	-----	-----	-----	-----	-----
<i>Rh. zambeziensis</i> (KY457509)	-----	-----	-----	-----	-----
<i>Rh. zambeziensis</i> (KY457507)	-----	-----	-----	-----	-----
18S F	-----	-----	-----	-----	-----
18S R	-----	-----	-----	-----	-----
	410	420	430	440	450
<i>Rh. appendiculatus</i> (KY457501)	C T C T T T T G A G	G C C C C G T A A T	T G A A A T G A G T	A C A C T C T A A A	T C C T T T A A C G
<i>Rh. appendiculatus</i> (KY457500)	-----	-----	-----	-----	-----
<i>Rh. evertsi</i> (KY457503)	-----	-----	-----	-----	-----
<i>Rh. evertsi</i> (KY457502)	-----	-----	-----	-----	-----
<i>Rh. maculatus</i> (KY457505)	-----	-----	-----	-----	-----
<i>Rh. maculatus</i> (KY457504)	-----	-----	-----	-----	-----
<i>Rh. microplus</i> (KY457506)	-----	-----	-----	-----	-----
<i>Rh. simus</i> (KY457508)	-----	-----	-----	-----	-----
<i>Rh. zambeziensis</i> (KY457509)	-----	-----	-----	-----	-----
<i>Rh. zambeziensis</i> (KY457507)	-----	-----	-----	-----	-----
18S F	-----	-----	-----	-----	-----
18S R	-----	-----	-----	-----	-----
	460	470	480	490	500
<i>Rh. appendiculatus</i> (KY457501)	A G G A T C A A T T	G G A G G G C A A G	T C T G G T G C C A	G C A G C C G C G G	T A A T T C C A G C
<i>Rh. appendiculatus</i> (KY457500)	-----	-----	-----	-----	-----
<i>Rh. evertsi</i> (KY457503)	-----	-----	-----	-----	-----
<i>Rh. evertsi</i> (KY457502)	-----	-----	-----	-----	-----
<i>Rh. maculatus</i> (KY457505)	-----	-----	-----	-----	-----
<i>Rh. maculatus</i> (KY457504)	-----	-----	-----	-----	-----
<i>Rh. microplus</i> (KY457506)	-----	-----	-----	-----	-----

Rh. simus (KY457508)
Rh. zambeziensis (KY457509)
Rh. zambeziensis (KY457507)
185 F
185 R

510 520 530 540 550

Rh. appendiculatus (KY457501)
Rh. appendiculatus (KY457500)
Rh. evertsi (KY457503)
Rh. evertsi (KY457502)
Rh. maculatus (KY457505)
Rh. maculatus (KY457504)
Rh. microplus (KY457506)
Rh. simus (KY457508)
Rh. zambeziensis (KY457509)
Rh. zambeziensis (KY457507)
185 F
185 R

560 570 580 590 600

Rh. appendiculatus (KY457501)
Rh. appendiculatus (KY457500)
Rh. evertsi (KY457503)
Rh. evertsi (KY457502)
Rh. maculatus (KY457505)
Rh. maculatus (KY457504)
Rh. microplus (KY457506)
Rh. simus (KY457508)
Rh. zambeziensis (KY457509)
Rh. zambeziensis (KY457507)
185 F
185 R

610 620 630 640 650

Rh. appendiculatus (KY457501)
Rh. appendiculatus (KY457500)
Rh. evertsi (KY457503)
Rh. evertsi (KY457502)
Rh. maculatus (KY457505)
Rh. maculatus (KY457504)
Rh. microplus (KY457506)
Rh. simus (KY457508)
Rh. zambeziensis (KY457509)
Rh. zambeziensis (KY457507)
185 F
185 R

660 670 680 690 700

Rh. appendiculatus (KY457501)
Rh. appendiculatus (KY457500)
Rh. evertsi (KY457503)
Rh. evertsi (KY457502)
Rh. maculatus (KY457505)
Rh. maculatus (KY457504)
Rh. microplus (KY457506)
Rh. simus (KY457508)
Rh. zambeziensis (KY457509)
Rh. zambeziensis (KY457507)
185 F
185 R

710 720 730 740 750

Rh. appendiculatus (KY457501)
Rh. appendiculatus (KY457500)
Rh. evertsi (KY457503)
Rh. evertsi (KY457502)

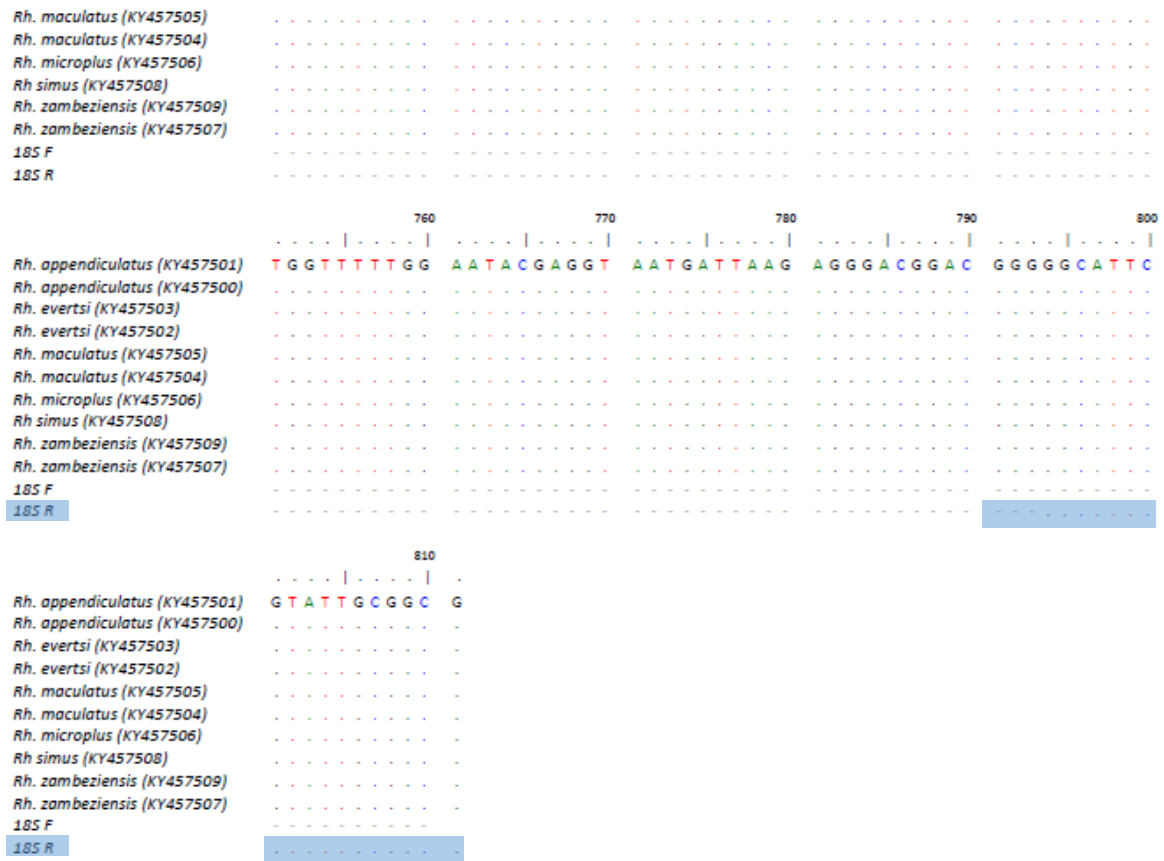


Figure 6.01 18S primer binding positions for *Rhipicephalus* species ticks

Appendix 6.3 – ITS2 primer binding positions for *Rhipicephalus* species ticks

	10	20	30	40	50
<i>Rh. appendiculatus</i> (KY457501)	C A T T G C G G C C	T T G G G T C T T C	C C T T G G C T T C	G T C T G T C T G A	G G G T C G G A T C
<i>Rh. appendiculatus</i> (KY457500)					
<i>Rh. evertsi</i> (KY457503)					
<i>Rh. evertsi</i> (KY457502)					
<i>Rh. maculatus</i> (KY457505)					
<i>Rh. maculatus</i> (KY457504)					
<i>Rh. microplus</i> (KY457506)					
<i>Rh. simus</i> (KY457508)					
<i>Rh. zambeziensis</i> (KY457509)					
<i>Rh. zambeziensis</i> (KY457507)					
ITS 2 F					
ITS 2 R					
ITS2 F Int					
ITS2 R Int					
	60	70	80	90	100
<i>Rh. appendiculatus</i> (KY457501)	A C A T A T C A A G	A G A G A C T T C G	G C G C A C A A G G	G A A C G T G G G C	C G T C G A C T C G
<i>Rh. appendiculatus</i> (KY457500)					
<i>Rh. evertsi</i> (KY457503)					
<i>Rh. evertsi</i> (KY457502)					
<i>Rh. maculatus</i> (KY457505)					
<i>Rh. maculatus</i> (KY457504)					
<i>Rh. microplus</i> (KY457506)		C			
<i>Rh. simus</i> (KY457508)				A	
<i>Rh. zambeziensis</i> (KY457509)			A		
<i>Rh. zambeziensis</i> (KY457507)			A		
ITS 2 F					
ITS 2 R					
ITS2 F Int					
ITS2 R Int					
	110	120	130	140	150
<i>Rh. appendiculatus</i> (KY457501)	T T T T G A C C G C	G T C G G C A T C A	T G G A C A G C A C	G T G G A A C G C A	- A T A G C A C -
<i>Rh. appendiculatus</i> (KY457500)					
<i>Rh. evertsi</i> (KY457503)			C		
<i>Rh. evertsi</i> (KY457502)			C		
<i>Rh. maculatus</i> (KY457505)					
<i>Rh. maculatus</i> (KY457504)					
<i>Rh. microplus</i> (KY457506)		A	C		
<i>Rh. simus</i> (KY457508)			C		
<i>Rh. zambeziensis</i> (KY457509)		T			
<i>Rh. zambeziensis</i> (KY457507)		T			
ITS 2 F					
ITS 2 R					
ITS2 F Int					
ITS2 R Int					
	160	170	180	190	200
<i>Rh. appendiculatus</i> (KY457501)	- G G C C G A C -	- T G C C G C T C	G A A G -	- G C G A G A C G G	C G G C G A - C
<i>Rh. appendiculatus</i> (KY457500)					
<i>Rh. evertsi</i> (KY457503)		A			
<i>Rh. evertsi</i> (KY457502)		A			
<i>Rh. maculatus</i> (KY457505)		A			
<i>Rh. maculatus</i> (KY457504)		A			
<i>Rh. microplus</i> (KY457506)		A			
<i>Rh. simus</i> (KY457508)	A	A			
<i>Rh. zambeziensis</i> (KY457509)		A G			
<i>Rh. zambeziensis</i> (KY457507)		A G			
ITS 2 F					
ITS 2 R					
ITS2 F Int					
ITS2 R Int					
	210	220	230	240	250
<i>Rh. appendiculatus</i> (KY457501)	C G C C G T G T C G	G A G - C T C G A C	- - C G A A A C G	G G G - C G C C C G	A C A G C A C T G A
<i>Rh. appendiculatus</i> (KY457500)					

<i>Rh. evertsi</i> (KY457503)	. . T . . . C C	 G . A T . . . T . . .
<i>Rh. evertsi</i> (KY457502)	. . T . . . C C	 G . A T . . . T . . .
<i>Rh. maculatus</i> (KY457505)	. . T . . . C C T A T	 A T
<i>Rh. maculatus</i> (KY457504)	. . T . . . C C T A T	 A T
<i>Rh. microplus</i> (KY457506)	. . T . . . C A C . A	 G . A T . . . T . . .
<i>Rh. simus</i> (KY457508)	. . T . . . C G C	 A A T
<i>Rh. zambeziensis</i> (KY457509)	 A	 A
<i>Rh. zambeziensis</i> (KY457507)	 A	 A
ITS 2 F		
ITS 2 R		
ITS2 F Int		
ITS2 R Int		
	260 270 280 290 300	
<i>Rh. appendiculatus</i> (KY457501)	G G - T G A C G G C A C C A - T C A A A G A C C G C G C A G G - A C T C G A A T C G G A A A G A	
<i>Rh. appendiculatus</i> (KY457500)		
<i>Rh. evertsi</i> (KY457503)	. . T . A T A . G A C T . T G A .	
<i>Rh. evertsi</i> (KY457502)	. . T . A T A . G A C T . T G A .	
<i>Rh. maculatus</i> (KY457505)	 T A A C T T A .	
<i>Rh. maculatus</i> (KY457504)	 T A A C T T A .	
<i>Rh. microplus</i> (KY457506)	. . A . . T T C G T T G A C T G G .	
<i>Rh. simus</i> (KY457508)	. . G . . T G A G G T	
<i>Rh. zambeziensis</i> (KY457509)		 G
<i>Rh. zambeziensis</i> (KY457507)		 G
ITS 2 F		
ITS 2 R		
ITS2 F Int		
ITS2 R Int		
	310 320 330 340 350	
<i>Rh. appendiculatus</i> (KY457501)	 G C C T G C A G G G A A A G C G C G G T C G A - G G T G T C C G A C T C C T C T C T G C G A C	
<i>Rh. appendiculatus</i> (KY457500)		
<i>Rh. evertsi</i> (KY457503)	A T C T G . G	
<i>Rh. evertsi</i> (KY457502)	A T C T G . G	
<i>Rh. maculatus</i> (KY457505)	A A G	A G . G
<i>Rh. maculatus</i> (KY457504)	A A G	A G . G
<i>Rh. microplus</i> (KY457506)	A T	T G . G T
<i>Rh. simus</i> (KY457508)		A G . G T
<i>Rh. zambeziensis</i> (KY457509)		
<i>Rh. zambeziensis</i> (KY457507)		
ITS 2 F		
ITS 2 R		
ITS2 F Int		
ITS2 R Int		
	360 370 380 390 400	
<i>Rh. appendiculatus</i> (KY457501)	C G A G A G C G C G A G A T A T - G C G A G A G A C T C G G A A G C G C C A C T T G - A A C G C A C	
<i>Rh. appendiculatus</i> (KY457500)		
<i>Rh. evertsi</i> (KY457503)	. . G A . C . T A C A T T	
<i>Rh. evertsi</i> (KY457502)	. . G A . C . T A C A T T	
<i>Rh. maculatus</i> (KY457505)	 T A . C	 A G
<i>Rh. maculatus</i> (KY457504)	 T A . C	 A G
<i>Rh. microplus</i> (KY457506)	. . G . C . . A G C C . A C A T	
<i>Rh. simus</i> (KY457508)	 G A . C	 C A G
<i>Rh. zambeziensis</i> (KY457509)	. . G	
<i>Rh. zambeziensis</i> (KY457507)	. . G	
ITS 2 F		
ITS 2 R		
ITS2 F Int		
ITS2 R Int		
	410 420 430 440 450	
<i>Rh. appendiculatus</i> (KY457501)	G G A G A G C G A A A G C C G G C C A G C T A G C T C G A A G T G	
<i>Rh. appendiculatus</i> (KY457500)		
<i>Rh. evertsi</i> (KY457503)	. . T G A A C A C G G G	G . A G C T C C T C C A G C C G T . C .
<i>Rh. evertsi</i> (KY457502)	. . T G A A C A C G G G	G . A G C T C C T C C A G C C G T . C .
<i>Rh. maculatus</i> (KY457505)	. . T G C A T A C G G G	G T C C C C C A G C C G T . C .
<i>Rh. maculatus</i> (KY457504)	. . T G C A T A C G G G	G T C C C C C A G C C G T . C .

Figure 1 displays the ITS2 sequence alignment of *Rh. appendiculatus* (KY457501), *Rh. appendiculatus* (KY457500), *Rh. evertsi* (KY457503), *Rh. evertsi* (KY457502), *Rh. maculatus* (KY457505), *Rh. maculatus* (KY457504), *Rh. microplus* (KY457506), *Rh. simus* (KY457508), *Rh. zambeziensis* (KY457509), and *Rh. zambeziensis* (KY457507) across the ITS2 region. The alignment is shown in three panels, with positions 460-500, 510-550, and 560-600. The sequences are color-coded: A (green), G (blue), C (red), and T (black). The alignment shows high similarity between the sequences, with some variations highlighted in the original image. The ITS2 region is divided into two main parts: the ITS2 F int (ITS2 F internal) and the ITS2 R int (ITS2 R internal). The ITS2 F int is located between positions 460 and 500, and the ITS2 R int is located between positions 510 and 550. The ITS2 R int is further divided into two sub-regions: the ITS2 R int (ITS2 R internal) and the ITS2 R int (ITS2 R internal). The alignment shows that the ITS2 R int (ITS2 R internal) is highly conserved across all species, while the ITS2 R int (ITS2 R internal) shows more variation.

	660	670	680	690	700
ITS 2 F					
ITS 2 R					
ITS2 F int					
ITS2 R int					
<i>Rh. appendiculatus</i> (KY457501)	T G G T A T - G C	G G G G A G A A C T	G C A C G G C G A C	T C G T C G C G A A	G C G G A A A A -
<i>Rh. appendiculatus</i> (KY457500)	-	-	-	-	-
<i>Rh. evertsi</i> (KY457503)	C - C C - T T	-	-	C -	- G - G -
<i>Rh. evertsi</i> (KY457502)	C - C C - T T	-	-	C -	- G - G -
<i>Rh. maculatus</i> (KY457505)	C - C C -	- G - C -	- G -	-	-
<i>Rh. maculatus</i> (KY457504)	C - C C -	- G - C -	- G -	-	-
<i>Rh. microplus</i> (KY457506)	C - T G A T -	- A - C - G	- G - A -	- T - C -	-
<i>Rh. simus</i> (KY457508)	C - C C - T -	-	- G -	-	- A A
<i>Rh. zambeziensis</i> (KY457509)	- - G -	-	-	-	-
<i>Rh. zambeziensis</i> (KY457507)	- G -	-	-	-	-
ITS 2 F					
ITS 2 R					
ITS2 F int					
ITS2 R int					
	710	720	730	740	750
<i>Rh. appendiculatus</i> (KY457501)	- T G T C T C C C G	C A C G A G T T G G	C G C C A C G T T G	G C G A A G C T - T	C A A G C G T T C C
<i>Rh. appendiculatus</i> (KY457500)	-	-	-	-	-
<i>Rh. evertsi</i> (KY457503)	-	- T -	- A A -	-	- G - T
<i>Rh. evertsi</i> (KY457502)	-	- T -	- A A -	-	- G - T
<i>Rh. maculatus</i> (KY457505)	A -	- T -	- C -	- T C -	-
<i>Rh. maculatus</i> (KY457504)	A -	- T -	- C -	- T C -	-
<i>Rh. microplus</i> (KY457506)	- A -	-	- A A -	-	- A - G - T
<i>Rh. simus</i> (KY457508)	A C -	- T - C -	- A -	- C - C -	- G -
<i>Rh. zambeziensis</i> (KY457509)	-	- C -	- T -	-	- G -
<i>Rh. zambeziensis</i> (KY457507)	-	- C -	- T -	-	- G -
ITS 2 F					
ITS 2 R					
ITS2 F int					
ITS2 R int					
	760	770	780	790	800
<i>Rh. appendiculatus</i> (KY457501)	C G T C G T A G T C	C G C C G T C G G T	C C A A G T G C T T	C G C A G T T C C C	G T C C C G T A C A
<i>Rh. appendiculatus</i> (KY457500)	-	-	-	-	-
<i>Rh. evertsi</i> (KY457503)	-	-	-	-	- T -
<i>Rh. evertsi</i> (KY457502)	-	-	-	-	- T -
<i>Rh. maculatus</i> (KY457505)	-	-	-	-	- T -
<i>Rh. maculatus</i> (KY457504)	-	-	-	-	- T -
<i>Rh. microplus</i> (KY457506)	-	-	- T -	-	- T -
<i>Rh. simus</i> (KY457508)	-	-	-	-	- G -
<i>Rh. zambeziensis</i> (KY457509)	-	-	-	-	- T -
<i>Rh. zambeziensis</i> (KY457507)	-	-	-	-	- T -
ITS 2 F					
ITS 2 R					
ITS2 F int					
ITS2 R int					
	810	820	830	840	850
<i>Rh. appendiculatus</i> (KY457501)	A A A - C T G G G	C C A C T C C A G T	T G G G G C G G G G	G C G A C G C T A C	A C G A G A C G A T
<i>Rh. appendiculatus</i> (KY457500)	-	-	-	-	-
<i>Rh. evertsi</i> (KY457503)	- A -	-	-	-	-
<i>Rh. evertsi</i> (KY457502)	- A -	-	-	-	-
<i>Rh. maculatus</i> (KY457505)	-	-	-	-	-
<i>Rh. maculatus</i> (KY457504)	-	-	-	-	-
<i>Rh. microplus</i> (KY457506)	- A A -	-	-	-	-
<i>Rh. simus</i> (KY457508)	-	-	-	-	-
<i>Rh. zambeziensis</i> (KY457509)	-	-	-	-	-
<i>Rh. zambeziensis</i> (KY457507)	-	-	-	-	-
ITS 2 F					
ITS 2 R					
ITS2 F int					
ITS2 R int					



Figure 6.02 ITS2 primer binding positions for *Rhipicephalus* species ticks

Appendix 6.4 - UP AEC ethical clearance certificate – H0007-17



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Animal Ethics Committee

PROJECT TITLE	Zoonotic rickettsioses in SA: Diagnostic, taxonomic and epidemiological aspects
PROJECT NUMBER	H007-17
RESEARCHER/PRINCIPAL INVESTIGATOR	A Trataris-Rebisz

STUDENT NUMBER (where applicable)	U_23085372
DISSERTATION/THESIS SUBMITTED FOR	PhD

ANIMAL SPECIES	Rodents	Cats	Dogs
NUMBER OF ANIMALS	385	105	98
Approval period to use animals for research/testing purposes			June 2017-August 2018
SUPERVISOR	Prof. W Markotter		

KINDLY NOTE:

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

APPROVED	Date 2017
CHAIRMAN: UP Animal Ethics Committee	Signature 

S4285-15

