Transmission dynamics of *Bartonella* in invasive *Rattus* from South Africa

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Transmission dynamics of *Bartonella* in invasive *Rattus* from South Africa

by

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This work is dedicated to my parents, Lillian and Ntsengeni Lithole for nurturing a dream they saw in me as a little girl.

clipArthut

Endurance produces character, and

Character produces hope!!

Romans5:4

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GENERAL ABSTRACT

Bartonellosis encompasses a broad range of diseases and clinical symptoms caused by bacteria of the species-rich genus, *Bartonella*. Three discrete species have been identified in *Rattus* from South Africa, of which one, *B. elizabethae* has known zoonotic potential. Brettschneider *et al*. (2012) developed a model to study the dynamics of *Bartonella* transmission related to host and ectoparasite vector aspects. However, the model was based on limited data for a number of crucial biological parameters and was unable to fully explain the differential infection rates observed in invasive rats. The present study attempts to obtain empirical estimates for key biological parameters that had to be estimated for that model, based on general rodent data, rather than *Rattus*-specific data. This was achieved using a molecular approach in which *gltA* and *nuoG* gene amplification was used to assess *Bartonella* prevalence in invasive *Rattus*, their associated ectoparasites and in, foetuses of *Bartonella*-positive pregnant female rats. Results revealed a *Bartonella* genome prevalence of 70.2% in pregnant females and 55.2% in foetuses. Similar *Bartonella* species were isolated from *Rattus* mothers and their foetuses, confirming vertical transmission of this bacterium in this rodent genus for the first time. A total of 1400 *Rattus*-associated ectoparasites (mites, ticks, flies and lice) were collected and identified morphologically. Molecular screening of a subset n=108 of *Xenopsylla brasiliensis* and *X. cheopis* fleas and *Haemaphysalis elliptica* ticks recovered a *Bartonella* genome prevalence of 84.3% in fleas and 0% in ticks. The corresponding *Bartonella* prevalence for the *Rattus* hosts from which the ectoparasites were obtained, was 54.1%. Two rodent-associated *Bartonella* species with known zoonotic potential were identified in this study, namely *B. elizabethae* and *B. tribocorum*. The results of this study can be used to: (i) further refine the epidemiological mathematical model developed by Brettschneider *et al* (2012), (ii) contribute to the existing body of knowledge on *Bartonella* infections in Africa, and (iii) assist medical, veterinary, municipal, conservation, and agricultural authorities in formulating suitable management strategies associated with problem rodent pests.

Keywords: *Bartonella, Rattus*, zoonotic disease, commensal rodents, invasive, *gltA* *nuoG*, urban/peri-urban, South Africa

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DECLARATION

I, the undersigned hereby declare that the dissertation, which I hereby submit for the degree of Master of Science (Zoology) 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.

SIGNATURE……

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LIST OF ABBREVIATIONS

%	Percentage
° C	Degree Celsius
µl	Microliter
µM	Micromolar
BI	Bayesian Inference
BIC	Bayesian Information Criterion
BLAST	Basic Local Alignment Tool
CO_2	Carbon dioxide
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotides
EIA	Enzyme linked immunoassay
gltA	Citrate Synthase Gene
IFA	Indirect Florescent antibody
MCMC	Markov Chain Monte Carlo
MEGA	Molecular Evolutionary Genetics Analysis
ML	Maximum Likelihood
mtDNA	Mitochondrial Deoxyribonucleic Acid
NCBI	National Centre for Biotechnology Information
NJ	Neighbour Joining
nuoG	NADH dehydrogenase gamma subunit
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid

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DISCLAIMER

This dissertation consists of a series of chapters that have been prepared as stand-alone manuscripts for subsequent submission for publication purposes. Consequently, unavoidable overlaps and/or repetitions may occur between chapters.

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Chapter 1

1.1 General Introduction

The genus *Rattus* comprises of approximately 66 species allocated to the subfamily Murinae within the Family Muridae, under the Order Rodentia which represents the largest mammalian Order (Musser and Carlenton 2005). Three of these species of *Rattus* occur in South Africa where they are all invasive. These include the black rat (*R. rattus*) native to the Indian peninsula, the brown rat (*R. norvegicus*) which is believed to be native to Papua New Guinea (Musser and Carlenton 2005) but whose precise origins are contentious (Amori and Clout 2003), and *R. tanezumi*, which is native to south-east Asia and has only recently been molecularly identified in South Africa (and Africa) (Bastos *et al.* 2005). *Rattus tanezumi* was first recorded to occur in four villages (Bloublommetjieskloof, Mapate, Nkomo-B, and Gaphoala) in Limpopo Provinces (Bastos *et al.* 2005), and then in the KwaZulu Natal Province (Taylor *et al.* 2008) of South Africa.

Currently, morphological distinction is only possible between the larger body-sized and ground-dwelling *R. norvegicus* and members of the smaller body-sized and arboreal *R. rattus* species complex (Amori and Clout 2003) that in South Africa comprises of *R. rattus* and *R. tanezumi* (Aplin et al. 2011). Due to the cryptic nature of *R. rattus* and *R. tanezumi*, molecular techniques are used to determine maternal species origin through nucleotide sequencing of a mitochondrial DNA (mtDNA) marker (Pagès *et al.* 2010; Aplin *et al.* 2011; Bastos *et al.* 2011). As introgression between the two members of the *R. rattus* species complex has been recorded at a number of localities (Chinen *et al*. 2005; Lack *et al.* 2012; Conroy *et al.* 2013), many argue against the continued recognition of two distinct species (Aplin *et al*. 2011; Conroy *et al.* 2013).

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Based on a recent global-scale genetic study by Aplin *et al.* (2011), it was proposed that *R. rattus* and *R. tanezumi* are biologically too similar to be considered as separate species, and these authors conservatively assigned lineages for each of the formerly recognized species within the *R. rattus* species complex rather than formally recognizing species. However, as introgression only occurs at sites at which the species have been introduced, many authors still follow the classical recognition of *R. tanezumi* (Lack *et al.* 2012) on the basis of the discrete maternal lineages that are recovered using mtDNA. We opt in this study to do the same and will be using this genomic molecule to assign *R. rattus* and *R. tanezumi* mitotype (mitochondrial type) designations to the two members of the *R. rattus* complex that are present in South Africa.

Members of the genus *Rattus* currently maintain an almost worldwide distribution due to increased travel (*via* rail, ships, trucks and aeroplanes) and favourable living conditions created by the presence of humans (Chinen *et al.* 2005; Taylor *et al.* 2008). The close association of *Rattus* with humans has been successful primarily due to their high levels of adaptability, small body size and their ability to feed on a wide range of food types (Bastos *et al.* 2011). Ultimately, this association is of interest because of the economic cost incurred through damage to agricultural products, infrastructure and transmission of diseases both to humans and livestock (Bastos *et al.* 2005; Chinen *et al.* 2005; Davis *et al.* 2005)

Outbreaks of zoonotic diseases are linked with fluctuations in abundance of rodent reservoir populations (Mills and Childs 1998; Heyman *et al*. 2001; Olsson *et al.* 2003; Rose *et al*. 2003; Meheretu *et al.* 2013). Members of the genus *Rattus* have been linked to pathogens (viruses, bacteria,and nematodes respectively) such as hepatitis E virus, Hantavirus (causing Hantavirus pulmonary syndrome), *Yersinia pestis* (that causes bubonic plague), murine typhus (febrile illness), leptospira (Weil's disease, a severe form of leptospirosis), *Streptobaccillus* (rat bite fever) and the parasitic nematode *Angiostrongylus cantonesis* (which causes eosinophilic meningitis) (Hirano *et al.* 2003; Davis *et al.* 2005; Elliot 2007; Ngo 2008; Kimura *et al.* 2008; Taylor *et al.* 2008; Meerburg *et al.* 2009; Meng 2011; Himsworth *et al.* 2013). Of particular interest in the present study is that members of the genus *Rattus* have also been found to be reservoir hosts of the bacterial genus *Bartonella* (Birtles

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et al. 2001; Jardine *et al.* 2005; Mostert 2010; Brettschneider 2011) which causes diseases such as endocarditis, neuroretinitis, febrile illness and cat scratch disease (Himsworth *et al.* 2013).

Media reports on increased rat invasions and the incidence of rat bites in South Africa's urban and peri-urban areas are escalating (Julius 2013) justifying the need for more research on invasive rodents and their zoonotic potential in South Africa. Kajdacsi *et al.* (2013) reported that slum-dwelling expansion in urban centres is increasing the influx of *R. norvegicus* invasions into the city of Salvador in Brazil. In the same year, an increase in rat infestations was reported in the inner city neighbourhoods of British Columbia, Canada (Himsworth *et al.* 2013). This raises concern since there are little to no data describing invasive *Rattus* species populations (particularly ecological studies evaluating rodent demography) and their influence of disease dynamics in South Africa.

1.1.1 Bartonella

The genus *Bartonella* includes a variety of genetically-related gram-negative bacteria that parasitize erythrocytes or endothelial cells of a broad range of mammalian hosts (Schulein *et al*. 2001; Bai *et al.* 2008; Eicher and Dehio 2012). *Bartonella* infections in humans have been recognized since pre-Colombian times (Argan and Dolan 2002). In 1905, Alberto Barton identified *B. bacilliformis* within human erythrocytes, but this remained the sole member of the genus until early 1993 when *Rochalimaea quintana* and *R. henselae* were re-classified as members of the genus *Bartonella* (Brenner *et al*. 1993, Karen *et al.* 2000). *Bartonella* was identified as a discretionary intracellular bacterium when these three human pathogens (*B. quintana, B. henselae* and *B. bacilliformis*) were discovered *(*Alsmark *et al.* 2004). *Bartonella quintana* is the causative agent of trench fever, a disease that affected more than 1 million soldiers during World War I and is currently re-emerging among homeless people in urban Europe (Fournier *et al.* 2002). *Bartonella henselae* is the causative agent of cat scratch disease, a well-known zoonotic disease affecting cats and their owners worldwide. *Bartonella bacilliformis* is the causative agent for Oroya fever, an acute blood infection causing 'Peruvian warts', a chronic skin infection, in Andean South America (Koehler 1995; Argan and Dolan 2002; Alsmark *et al*. 2004).

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Currently, six rodent-borne *Bartonella* species or subspecies are recognized as human pathogens (Buffet *et al.* 2013). These are *B. washoensis* a causative agent for myocarditis (Breitschwerdt and Kordick 2000; Kosoy *et al.* 2003), *B. grahamii* detected in patients with neuroretinitis (Kerkhoff *et al.* 1999), *B. elizabethae* isolated from patients with endocarditis and febrile illness (Daly *et al.* 1993; Myint *et al.* 2011), *B. vinsonii arupensis* isolated from a cattle rancher suffering from neurological disorders and fever (Welch *et al.* 1999) and from febrile patients from Nepal (Myint *et al.* 2011), *B. tamiae* isolated from patients suffering from myalgia, fatigue, headache, maculopapular rash, fever and pterigium (Kosoy *et al.* 2008; Myint *et al.* 2011) and endocarditis and lastly *B. tribocorum* which was isolated from patients with febrile illness in Thailand (Kosoy *et al*. 2010).

Bartonella infection is concurrent with immunological suppression of the host where the disease can either cause symptoms or in some cases remain asymptomatic not showing any clinical signs of infection in the host species (Regnery *et al.* 1992; O'Reilly *et al.* 1999; Trataris 2012). This bacterium has the ability to infect a wide range of mammalian hosts from humans, cattle, deer, lions, rabbits, wild dogs, porpoises, domestic cats and dogs, to rats and mice (Jacomo *et al.* 2002; Maggi *et al.* 2005b; Brettschneider 2011). Thirty-four *Bartonella* species have been identified to date and with increased interest in this re-emerging disease, new species are regularly being discovered (Buffet *et al.* 2013; Rasis *et al*. 2014). Table 1 below shows different *Bartonella* infection prevalences detected in South African *Rattus* species to date.

Table 1 *Bartonella* spp. Detected in South African *Rattus* spp. to date.

	Percent (%) *Bartonella* infection prevalence in South Africa			
Authors	Average percent (%) prevalence for the genus in South Africa	*R. rattus*	*R. tanezumi*	*R. norvegicus*
Mostert 2009	15.4%	6.8%	4.7%	39.0%
Brettschneider 2010	16.05%	5.55%	7.34%	27.2%
Trataris *et al.* 2012	25.0%	-	-	-

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1.1.2 Transmission

Bartonellosis is a vector-borne disease, where infections are transmitted by blood-feeding arthropods (Bai 2009) such as fleas, ticks and body lice (Fournier *et al.* 2002; Boulouis *et al.* 2005; Mostert 2010; Brettschneider 2011; Himsworth *et al.* 2013). Transmission occurs either by bite (anterior station transmission) or through fecal contamination of the bite site (posterior station transmission) (Abbot *et al*. 2007).

Vertical transmission has also been suggested as a potential mechanism by which infection can be maintained within either a vector or host population (Kosoy *et al*. 1998). Chomel (2011) found no evidence of vertical transmission of *Bartonella* in rodent fleas. Abbot *et al.* (1997) also found that kittens born to bacteraemic queens were abacteraemic at birth and remained abacteraemic through to weaning stage.

However, vertical transmission of *Bartonella* in arthropod vectors has been suggested after *Bartonella* DNA was detected in flea reproductive tissues (Brinkerhoff *et al.* 2010). Transplacental transmission of *Bartonella birtlesii* was also reported in BABLB/c mice, although none of the living offspring in the study were bacteraemic (Bloulois *et al.* 2001). In addition, *Bartonella* was isolated from neonates and embryos of naturally infected rodents in North America, revealing a 48% vertical transmission rate (Kosoy *et al.* 1998). Due to the latter, vertical transmission of *Bartonella* is suspected to occur in some species of rodents, including members of the genus *Rattus.*

1.1.3 Examples of recent human clinical cases caused by Bartonella henselae

Although limited, below are examples of recent *Bartonella* cases.

Case 1: Matias *et al*. (2013) - A 71 year old cancer patient remained under surveillance after chemotherapy. He developed an enlarged lymph node within three years of surveillance. The biopsy revealed a necrotising and abscessed granulomatous lymphadenitis suggesting that he could also be suffering from cat scratch disease. The serology for *B. henselae* was positive and the patient was

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treated for cat scratch disease. The patient admitted to prior exposure to cats and also confirmed to being scratched by one.

Case 2: Khalighi *et al.* (2013) - Diagnosis of *Bartonella* endocarditis was made using polymerase chain reaction (PCR) in an 18–year old Hispanic woman's blood after she presented with a 3-month history of fatigue, weight loss, anaemia and acute kidney injury. It took only one month prior to her exposure to cats to start showing symptoms of intermittent fevers, gross haematuria and frothy urine.

Case 3: Im *et al.* (2013) - Culturing, serological and conventional PCR test were used to investigate a case of a 73-year-old woman with a persistant fever in South Korea. On admission, her body temperature was 39˚ C, pulse rate of 90/min, blood pressure of 120/80 mm Hg and a respiratory rate of 18/min. The woman admitted to no prior exposure to cats; however, the isolated bacterium found in her blood was 98% identical to that of *B. henselae* Houston-1 strain from Genbank. It is possible that her Infection by this *Bartonella* species could have been due to exposure to different arthropod vectors implicated in *Bartonella* transmission.

Case 4: Losch and Wank (2014) - *Bartonella* associated with gross angioedema of the tongue in a 49-year-old healthy looking man. The man was experiencing swelling attacks of the tongue, hands and arms. The swelling of the tongue was so severe that he felt as if he was being suffocated. A real time PCR method was used to find the cause of the disease and *Bartonella* was identified. The findings of this study strongly suggest that *B. henselae* was responsible for the life-threatening angioedema of the tongue.

All of the above cases were caused by the same causative agent yet had highly variable clinical presentations. This stresses the difficulty of clinical diagnosis and emphasises the importance of laboratory diagnosis. Pathogenic potential among *Bartonella* spp. genotypes may vary within a species' individual isolates and host species infected (Brunt *et al.* 2006). Diseases associated with *Bartonella* in humans vary according to the immune system of the host (Brunt *et al.* 2006); hence the different clinical presentations mentioned above. Therefore, *Bartonella* diagnosis cannot be based on clinical signs and symptoms only since this may yield misleading results; clinical diagnosis and laboratory diagnosis should be used concurrently.

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1.1.4 Methods of Bartonella *detection*

Bacteria within the genus *Bartonella* are slow-growing, macroaerophilic, pleomorphic, gram-negative rods that belong to the alpha–proteobacteria (Anderson and Neuman 1997; Jacomo *et al.* 2002). Due to their fastidious nature and also because *Bartonella* infections are most likely to be asymptomatic, this bacterium is difficult to work with. Different techniques have been used to study members of the genus *Bartonella* such as culturing, serology, and most recently, molecular techniques (Anderson and Neuman 1997; Doern 2000; Agan and Dolan 2002; Fenollar and Raoult 2004; Diederen *et al*. 2007 and Chomel *et al.* 2009).

Consequently, the type of specimen collected and the facilities and equipment available may influence the choice of laboratory procedures used to confirm *Bartonella* infection (Anderson and Neuman 1997).The identification of *Bartonella* is generally achieved by culturing and molecular methods such as polymerase chain reaction (PCR) and DNA sequencing, while clinical diagnosis of active and past infections relies mostly on serological tests and detection of antibodies (Doern 2000; Bermond *et al.* 2002; Gundi *et al.* 2004; Maillard *et al.* 2004; Duncan *et al.* 2007).

Culturing

Culturing techniques traditionally have formed the cornerstone of establishing an etiological diagnosis of infection in patients whose disease is due to bacteria, fungi, and mycobacteria (Doern 2000). Culturing of strict intracellular bacteria is delicate, and cell culture is an obligatory step for their isolation (Gouriet *et al.* 2005). The advantage of culturing is that the microorganism is available for propagation and extended sampling allowing for antimicrobial susceptibility testing and infection experiments. Furthermore, a positive culture remains the ‘gold-standard’ requirement for describing a new bacterial species (Doern 2000).

Members of the genus *Bartonella* have been found to grow best on freshly-prepared heart infusion agar containing 5-10% defribrinated rabbit, sheep and horse blood in 5% CO_2 environment at between 35° C and 37° C (Batterman *et al.* 1995;

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Anderson and Neuman 1997; Jacomo *et al.* 2002; Gundi *et al.* 2004; Gouriet *et al.* 2005; Duncan *et al.* 2007; Chomel *et al.* 2009). However, requisite expertise for performing cultures are often absent in many clinical laboratories and because cases are erratic, the necessary media and supplies can become out-dated before use (Doern 2000). For these reasons, culturing proves tedious and often proves unsuccessful.

Serology

Serological tests for the detection of *Bartonella* include indirect florescent antibody (IFA), enzyme-linked immunoassay (EIA) and immunoblot tests (Agan and Dolan 2002). These tests are used for clinical diagnoses of different pathogens. IFA assays are difficult to perform, requiring significant expertise to provide reproducible results whereas EIA is relatively easy (Agan and Dolan 2002). Despite this, IFA remains the most widely used assay for *B. henselae* and in some cases *B. quintana* (Sander *et al.* 2001; Agan and Dolan 2002; Mattar and Parra 2006). This is because *B. henselae* and *B. quintana* share a close phylogenetic relationship, which makes it difficult to differentiate between the two species by serological means (Agan and Dolan 2002). Serological tests also showed lack of specificity and/or sensitivity in patients with cat scratch disease (Bergmans *et al.* 1995). Because of this, the use of new molecular techniques for diagnosis of cat scratch disease in patients with superficial lymphadenopathy was suggested (Hansmann *et al.* 2005).

Molecular techniques

Molecular techniques offer high sensitivity, specificity, and the ability to differentiate *Bartonella* organisms at the highest level (Agan and Dolan 2002). These technological innovations of identification have heralded a new era with respect to diagnostic microbiology (Fenollar and Raoult 2004). There are several different molecular methods used in *Bartonella* identification and these include conventional and real-time PCR, restriction fragment length polymorphism (RFLP) analysis, southern blotting, gel and capillary electrophoresis, DNA-hybridization and genetic sequence analysis (Houpkian and Raoult 2001; Renesto *et al.* 2001; Fenollar and Raoult 2004; Colborn *et al.* 2010).

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The development of these different molecular techniques is linked with the development of online databases which help in analysis and interpretation of results (Fenollar and Raoult 2004). These include online databases such as Genomes Online Database (Bernal *et al.* 2001) and Genbank (Benson *et al.* 2005). Molecular techniques are rapid but require a great deal of accuracy in order to yield good results. For example, the right choice of primers for different gene regions, the use of negative controls, separation of pre- and post-PCR, avoidance of horizontal and vertical contamination and in some cases, the use of a positive control is necessary especially when dealing with real time PCR (Fenollar and Raoult 2004).

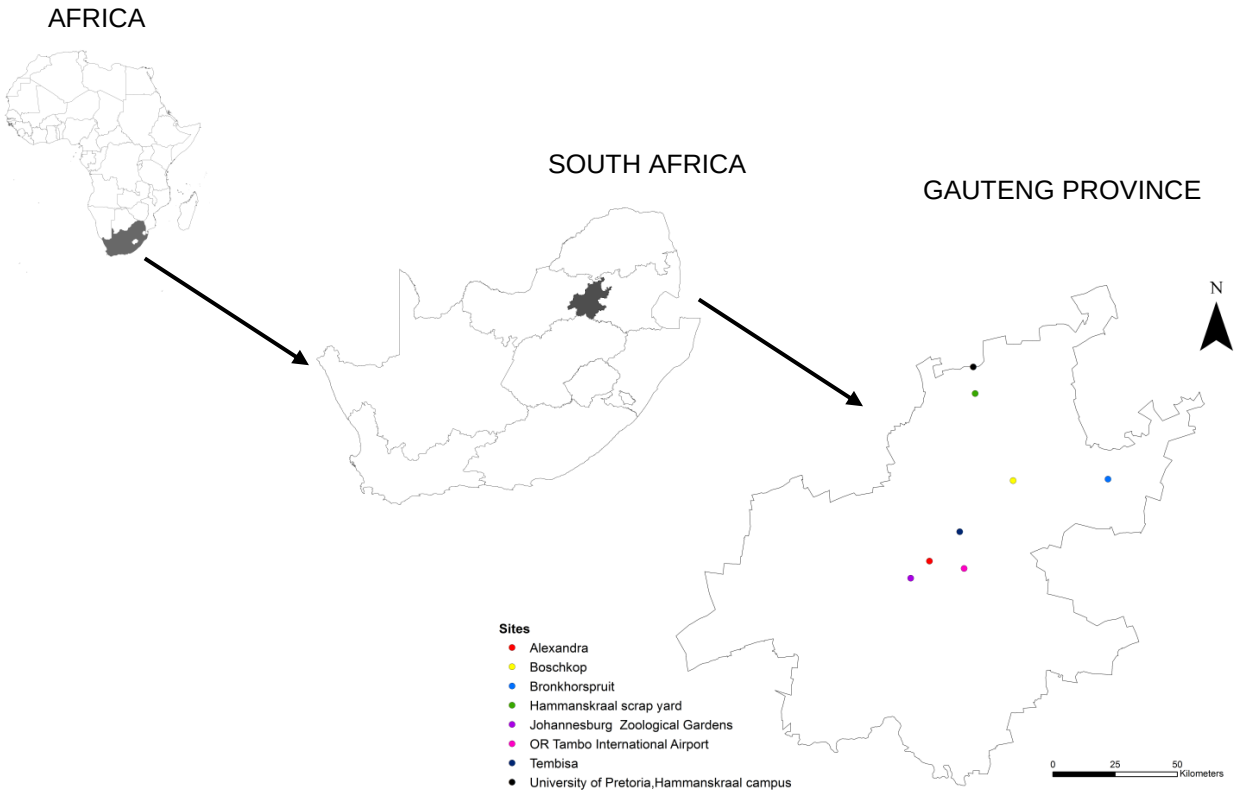

Figure 1: Map showing the study area where *Rattus* spp were sampled in Gauteng Province South Africa.

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1.2 Aims of study

The aim of the present study is to obtain empirical estimates for key biological parameters that influence *Bartonella* transmission in three invasive species of *Rattus* (*R. rattus, R. tanezumi and R. norvegicus*) from urban (informal settlements in the city centre) and peri-urban (settlements on the outskirts of the city) areas of Gauteng Province, South Africa (Figure 1). Invasive rat infestations are particularly prevalent in these urban and peri-urban environments (Brettschneider 2011; Himsworth *et al.* 2013, 2014; Kajdacsi *et al.* 2013). Thus by determining factors influencing transmission the mathematical epidemiological model developed by Brettschneider *et al.* (2012) can be refined and our understanding of *Bartonella* infections in South African *Rattus* species improved.

1.3 Research questions

The present study attempted to address the following research questions:

1. Is there vertical transmission of *Bartonella* in three invasive *Rattus* species from peri-urban and urban areas of Gauteng Province, South Africa?
2. Are there differences in *Bartonella* prevalence in *Rattus*-associated ectoparasites from peri-urban and urban areas of Gauteng Province, South Africa?

1.4 Research hypotheses

The following research hypotheses were tested in the present study:

Hypothesis 1:

H_0: There is no vertical transmission of *Bartonella* between female invasive *Rattus* species to their offspring in urban and peri-urban areas in Gauteng Province, South Africa.

H_a: There is vertical transmission of *Bartonella* between invasive female *Rattus* species to their offspring in urban and peri-urban areas in Gauteng Province, South Africa.

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Hypothesis 2:

H_0: There are no *Bartonella* infections in *Rattus*-associated ectoparasites from peri-urban and urban areas of Gauteng Province, South Africa.

H_a: There are *Bartonella* infections in *Rattus*-associated ectoparasites from peri-urban and urban areas of Gauteng Province, South Africa.

1.5 Relevance of study

Although members of the genus *Rattus* have been useful as model species for medical research (Schlick *et al*. 2006; Andersson *et al*. 2011) and for mapping human migration routes (Matisoo-Smith & Robins 2004; Matisoo-Smith & Robins 2009), the genus represents one of the most successful and destructive invasive mammalian genera. They invade native habitats reducing native fauna and have even caused extinction of some local species through predation and competition (Towns *et al.* 2006; Wyatt *et al.* 2008; Chiba 2010; Conroy *et al.* 2013).

Rattus species are also known to live commensally with humans and their small size enables them to achieve worldwide distribution by using human transportation systems (Bastos *et al.* 2011; Eisen and Cage 2012). They are pest species that destroy infrastructure and create food shortages for millions of people (Meerburg *et al.* 2009). *Rattus* species are of great concern because of an expanding human population that is estimated to reach 2.1 billion by 2030 (Taylor *et al.* 2008). This human population eruption may inevitably favour commensal rodent invasions, especially in informal settlements with inadequate infrastructure such as poor housing, sanitation and sewerage systems (Taylor *et al.* 2008; Costa *et al.* 2014). These expansive overcrowded slum-dwellings in urban areas will create abundant food resources and harbourage for these invasive rats (Kajdacsi *et al.* 2013).

Members of the genus *Rattus* are natural reservoirs of several pathogens responsible for a significant number of epidemic diseases in mammals (Smith and Carpenter 2006; Bastos *et al.* 2011; Heyman *et al.* 2013; Maheretu *et al.* 2013). An increase in *Rattus* invasions increases the risk of zoonotic disease transmission and it is therefore critical that we gain insights into the dynamics of potential pathogens

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and their host in order to formulate effective control measures for different diseases (Meyer 2003; Heyman *et al.* 2013).

Experiments can sometimes be difficult, expensive and/or unethical to undertake, hence mathematical modeling has become an important tool for studying infectious and vector-borne diseases (Diekman 1996; Roberts and Heesterbeek 2003; Graham *et al.* 2009; Brettschneider 2011; Huang and Takeuchi 2010). A major goal of theoretical and mathematical techniques in epidemiology is to develop an understanding of the interplay between variables that determine the course of infection within individuals and the variables that control the pattern of infections within communities (Nukes and Anderson 1988). Mathematical models can therefore encourage efficient use of limited resources and allow the targeting of control measures (Gilbert *et al.* 2014).

Consequently, Brettschneider *et al.* (2012) developed a compartmental epidemiological mathematical model using the SEI compartmental approach that incorporated 12 differential equations to investigate the dynamics of *Bartonella* infections in two invasive *Rattus* and two associated ectoparasite species in South Africa. The parameters of the model (Figure 2) are related to the transfer rates to and from the compartments determined by the vital dynamics of the populations (birth and deaths) as well as the course of the infection.

The model showed the need for more detailed biological data on *Bartonella* infections and the likely importance of the flea vector in the spread of this disease (Brettschneider *et al.* 2012). The present study attempts to provide *Rattus*-specific data that can be used to replace variables estimated from general/unrelated rodent data that was used in the original model. This information will contribute towards an increasing body of knowledge on vector-borne diseases in Africa, in turn giving direction on how to mitigate *Bartonella*, and other vector-borne infections. The present study may provide information that may assist municipal, conservation, and agricultural authorities in their attempt to formulate strategies to manage problems associated with rodent pests.

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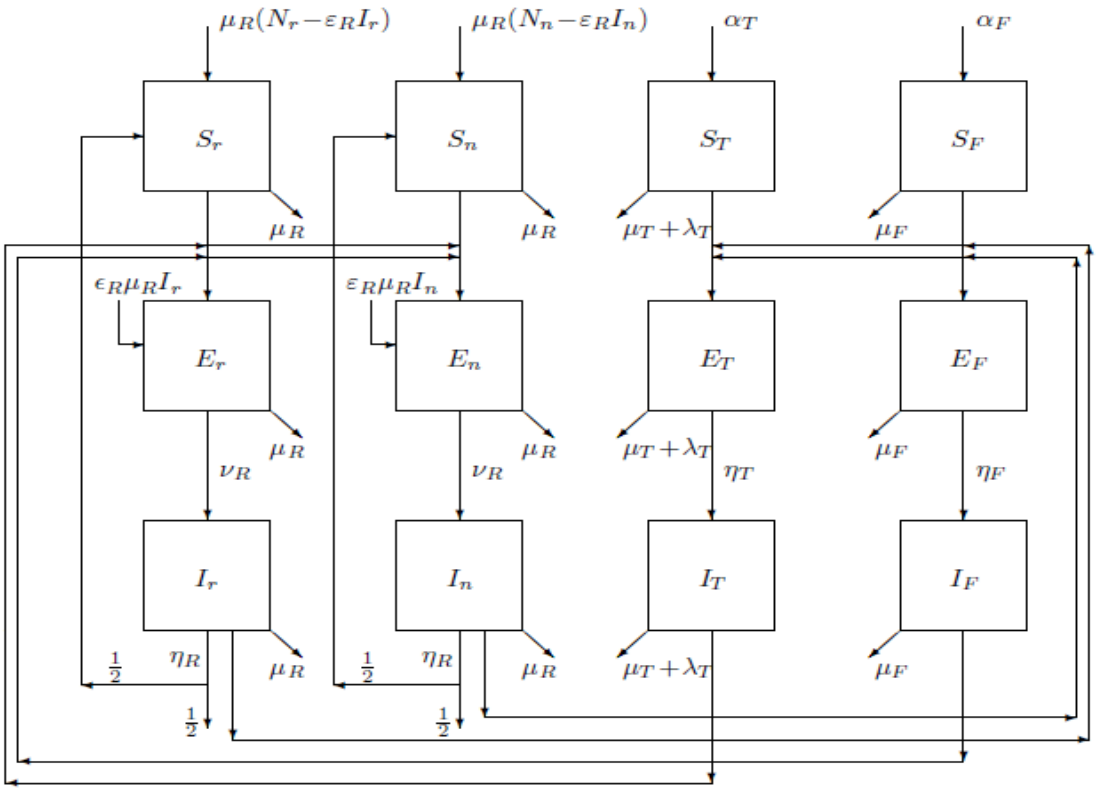

Figure 2. Compartmental structure and flow-diagram of *Bartonella* infections in *Rattus* (Adopted from Brettschneider 2011).

1.6 Study approach

The study was conducted in urban, peri-urban areas of Gauteng Province, South Africa. Rats were either snap-trapped or live-trapped by members of the public, and some were collected as part of an extermination program conducted by Johannesburg Municipality. Samples collected from Agriculture Research council (ARC), Rietondale campus in Pretoria and those from a previous study (Julius 2013) were also included. Specimens were collected under collection permit CPF6 0032 issued by the Department of Nature Conservation in Gauteng and Animal ethics permit ECO25-10 issued by the Animal Ethics Committee of University of Pretoria, South Africa. Permission to conduct research in terms of section 20 of the Animal Disease Act (Act No 35 of 1984) was granted by the Department of Agriculture,

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Forestry and Fisheries (Ref 13788). All permits and approvals are included in appendix 1.

Organ samples and embryos (where applicable) were dissected and stored at –20^{o} C, while all ectoparasites removed from dead animals were preserved in absolute ethanol. Animals and ectoparasites were identified morphologically where possible with the aid of identification keys and experts. A subset of *Rattus* specimens were identified molecularly by targeting the cytochrome *b* gene region in order to confirm morphological identifications. All voucher specimens were deposited in the Ditsong National Museum of Natural History (formerly the Transvaal Museum) of the Northern Flagship Institute (NFI), Pretoria, South Africa. Genomic DNA from organs of interest was extracted using Roche High Pure Extraction Kit (Roche Diagnostics) according to the manufacturer's protocol. The presence of *Bartonella* was tested targeting the citrate synthase (*gltA*) gene region (513bp & 722bp) and the NADH dehydrogenase gamma subunit (*nuoG*) gene region (346bp) (Norman *et al.* 1995; Bastos 2007; Colborn *et al*. 2010).

PCR reactions were prepared in a DNA-free hood subsequent to decontamination with UV light for 10 minutes. Contamination was prevented following the recommendations of Fenollar and Raoult (2004). PCR reactions were scored *Bartonella*-positive based on corresponding amplification of two gene regions, or by repeat amplification and sequencing of the same gene region. The Roche PCR product purification kit was used according to manufacturer's instructions to purify amplicons directly from the tube. Purified products were cycle sequenced using BigDye v. 3.1 terminator cycle-sequencing kit (Perkim-Elmer, Foster City, and U.S.A.) with forward and reverse primers, in separate reactions. Samples were run on an ABI 3130 sequencer and resulting sequence chromatograms were viewed and edited in Chromas program embedded in Mega 5 (Tamura *et al.* 2011). BLAST nucleotide searches were performed (www.ncbi.nm.nih.gov/blast) to identify *Bartonella* reference species sequences with the highest nucleotide sequence identity.

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1.7 Study outline

The first part of this study (Chapter 2) investigated vertical transmission of *Bartonella* in *R. norvegicus* females and their embryos. This species was targeted because of its abundance in informal settlements. Pregnant females and their embryos were screened for *Bartonella* presence and *Bartonella* species present in mothers were compared phylogenetically to those found in their embryos, in order to assess vertical transmission potential and rates. Chapter 3 assessed the prevalence and diversity of *Rattus*-associated ectoparasites and their *Bartonella* status. Ectoparasites evaluated included ticks, mites, fleas and lice which were identified to genus level and where possible to the species level. Different infection rates and phylogenetic relationships of *Bartonella* amongst ticks, mites, fleas and their host were determined. A general discussion, conclusions drawn from this study and recommendations for future studies are provided in Chapter 4.

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1.8 References

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Chapter 2

Molecular assessment of vertical transmission rates of *Bartonella elizabethae* and *B. tribocorum* in invasive *Rattus norvegicus* from Gauteng Province, South Africa

Abstract

Bartonella was detected in 40 of the 57 pregnant *Rattus norvegicus* females (70.2%) sampled in peri-urban and urban areas of Gauteng Province, South Africa. On dissection, nine individuals were found to have foetuses sufficiently large to allow for torso dissection. DNA extracted from the 87 foetuses collected from the nine *Bartonella*-positive mothers was tested for *Bartonella* genome presence using PCRs targeting the *gltA* and *nuoG* gene regions. It was found that two of the *Bartonella* positive mothers had foetuses that were *Bartonella* negative and that 48 of the 87 foetuses (55.2%) were positive. Nucleotide sequencing of positive amplicons confirmed the presence of *B. elizabethae* and *B tribocorum* in pregnant females, and that the strains carried by the mothers matched those of their foetuses. Phylogenetic analyses based on the *gltA* gene region confirmed that four *R. norvegicus* mothers and their foetuses were infected with *B. tribocorum* (99% bootstrap support) while one mother and its foetus clustered within the *B. elizabethae* lineage (92% bootstrap support). These findings suggest the possibility of vertical transmission being one of the mechanisms for maintaining *Bartonella* infections in the invasive *R. norvegicus* population and are of public health concern as the two *Bartonella* species detected in *R. norvegicus* have been linked to cases of endocarditis and febrile illness in humans.

Keywords: *Bartonella, Rattus norvegicus,* vertical transmission, zoonotic pathogens, commensal rodents, *gltA*, *nuoG*, South Africa

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2.1 Introduction

Emerging and re-emerging rodent-borne diseases have become a serious public health concern due to increasingly close associations between rodents and humans. Members of the genus *Rattus* are no exception, and are known to carry a wide range of pathogens that include zoonotic bacteria, viruses, and protozoans (Himsworth *et al*. 2013; Chapter 1). This genus represents one of the most successful and destructive invasive mammalian genera, destroying crops and infrastructure, and causing substantial economic losses (Meerburg *et al.* 2009; Meng 2011). Due to their close association with humans, this group of rodents have been used for mapping human migration routes (Matisoo-Smith and Robins 2004; Matisoo-Smith and Robins 2009) and their small size and broad diet enables them to invade many parts of the world (Aplin *et al.* 2011; Bastos *et al*. 2011). Three invasive *Rattus* species are known to occur in South Africa, namely, *R. rattus, R. norvegicus* and *R. tanezumi* (Bastos *et al.* 2011).

Human population increases are linked with an increase in informal settlements which are characterized by inadequate infrastructure such as poor housing and sewerage systems, and uncontrolled waste disposal. This kind of environment promotes *Rattus* species infestation due to abundant food resources and represents an ideal environment for invasive rodents to florish in (Himsworth *et al.* 2013; Kajdacsi *et al.* 2013; Costa *et al.* 2014). It is estimated that the global urban human population will increase to 2.1 billion by 2030 (Taylor *et al.* 2008). This will lead to increased human-rat contact, with a likely increase in the risk of zoonotic disease transmission, including *Bartonella*. Bartonellae are considered to be re-emerging pathogens, with zoonotic potential and are therefore of growing interest in medical and veterinary research (Agan and Dolan 2002; Jacomo *et al.* 2002; Maillard *et al.* 2006; Chomel *et al.* 2009; Breitschwerdt *et al.* 2010; Brettschneider *et al.* 2012; Buffet *et al.* 2013; Hayman *et al.* 2013; Darvishi *et al.* 2014; Mediannikov *et al.* 2014).

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Bartonella species are facultative, gram-negative intracellular bacteria belonging to the alpha 2 subgroup of the proteobacteria (Boulouis *et al*. 2005; Chomel 2006; Saisongkorch *et al.* 2009). These are fastidious bacteria that infect endothelial and erythrocyte cells of a wide range of mammalian hosts (Schulein *et al.* 2001; Eicher *et al.* 2012). There are approximately 34 *Bartonella* species recognized to date, with more continuously being discovered (Buffet *et al.* 2013; Rasis *et al.* 2014). The best known zoonotic species are *B. bacilliformis* which causes Carrion's disease, *B. quintana* which is the causative agent of trench fever and *B. henselae* an agent of cat scratch disease (Dehio 2008; Mediannikov *et al.* 2014).

Twenty of the recognised *Bartonella* species were initially or subsequently detected in wild rodents, with six of the rodent-borne *Bartonella* species known to have zoonotic potential, causing neuroretinitis, endocarditis, febrile illness, myocarditis, lymphadenitis, hepatitis and fever (Buffet *et al.* 2013). The six rodent-borne pathogenic *Bartonella* species include *B. elizabethae* (Daly *et al.* 1993), *B. grahamii* (Kerkhoff *et al*. 1999), *B. vinsonii arupensis* (Welch *et al.* 1999), *B. washoensis* (Kosoy *et al.* 2003), *B. tamiae* (Kosoy *et al.* 2008), and most recently *B. tribocorum,* however the latter species requires further investigation, (Kosoy *et al.* 2010).

Bartonella infection can either be symptomatic or asymptomatic depending on the infected mammalian host. The infection strategy of *Bartonella* in the blood stream of any mammalian reservoir is to establish a long-lasting intra-erythrocytic bacteremia (Chomel *et al.* 2009). In incidental hosts such as humans, the infection may result in immune suppression making the infected host vulnerable to opportunistic infections and may even cause the immune system to mistakenly attack healthy cells (Chomel *et al.* 2009).

Understanding the route of disease transmission is important for mitigation and prevention of intra- and inter-specific transmission in wild animals, livestock and humans (Hayman *et al.* 2013). Horizontal transmission of *Bartonella* is suspected to occur through biting, scratching, and contact with faeces of infected blood-sucking arthropods (Bai *et al.* 2009; Chomel *et al.* 2009). It has been shown that fleas, lice, sand flies and ticks can transmit *Bartonella* (Chomel *et al.* 2009). *Bartonella* has also

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been detected in some blood-sucking arthropods such as biting flies, Keds and mites; however, their vector competencies remain unknown (Chomel *et al.* 2009). Another suggested mode of *Bartonella* transmission is through vertical transmission (Breitschwerdt and Kordick 2000; Johnson *et al.* 2009, Breitschwerdt *et al*. 2010). Transplacental transmission of *Bartonella* was reported in BABLB/c mice (Boulouis *et al.* 2001) and in neonates and embryos of naturally infected rodents (*Sigmodon hispidus* and *Peromyscus leucopus)* in North America (Kosoy *et al.* 1998).

The aim of this study is to assess the prevalence and to determine the *Bartonella* species present in pregnant female *R. norvegicus* rats and their foetuses, sampled primarily from informal settlements in Gauteng Province, South Africa, in order to determine the potential for vertical transmission. This may assist in our understanding of the dynamics of *Bartonella* infection in this invasive commensal *Rattus* species and shed light on whether vertical transmission of *Bartonella* is another mechanism whereby bacterial maintenance is achieved in *Rattus* populations. The present study focused mainly on *R. norvegicus* because of its abundance in informal settlements.

2.2 Materials and Methods

2.2.1 Sample collection

Sampling of *Rattus* species targeted six localities in Gauteng Province, South Africa (Figure1; Table1). *Rattus* specimens were snap-trapped (Scientific Supa-Kill Kempton Park, South Africa), live-trapped by members of the public during an extermination program conducted by the Johannesburg Municipality, sampled by the Agriculture Research Council (ARC) in a rodent survey project, donated by members of the public, and sourced from banked specimens collected in previous studies (Bastos *et al.* 2011; Julius 2013; Table 1). Live-trapped *Rattus* specimens were baited with a mixture of peanut butter, oats and fish oil. All live-trapped *Rattus* samples were sacrificed using CO_2 and immediately bagged in individual bags. Three hundred and forty-two *Rattus* specimens were sampled between 2012 and 2013 during the course of this study whereas seven *Rattus* specimens were

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collected between 2006 and 2011 during previous studies (Mostert 2010; Brettschneider 2011; Julius 2013). The samples were obtained under collection permit CPF6 0032 issued by the Department of Nature Conservation in Gauteng and Animal ethics permit ECO25-10 approved by the Animal Ethics Committee of University of Pretoria, South Africa. Permission to conduct research in terms of section 20 of the Animal Disease Act (Act No 35 of 1984) was granted by the Department of Agriculture, Forestry and Fisheries (Ref 13788); Appendix 1.

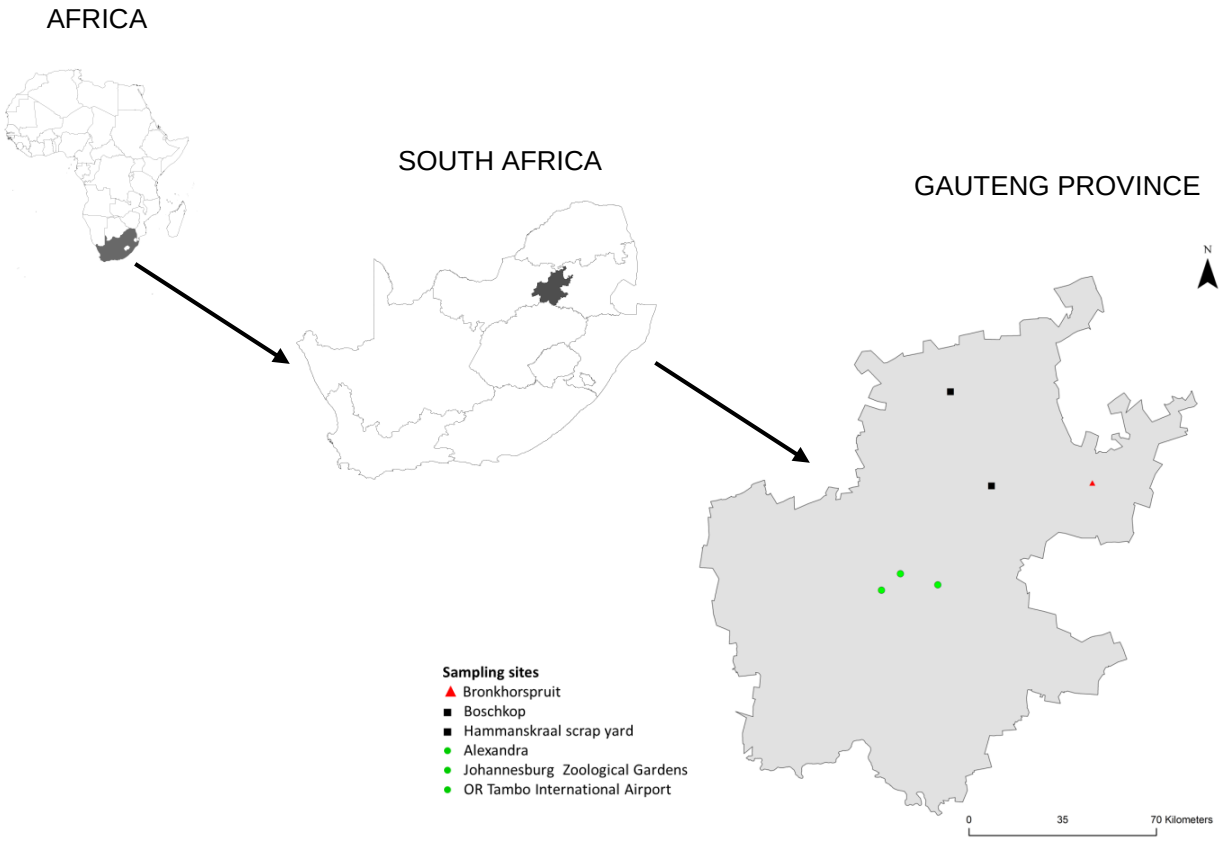

Figure 1. Map of Gauteng Province, South Africa showing sampling sites for *Rattus* spp. collected between 2010–2012.The green sampling points indicate areas where *R. norvegicus* was sampled.

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Table 1: Geographic coordinates of localities where pregnant *Rattus* specimens were sampled in Gauteng Province, South Africa.

Locality	*N*	Species name	latitude	longitude	Reference
Alexandra, Johannesburg	50	*R. norvegicus*	-26.623	28.577	This study
Boschkop, Pretoria	3	*R. tanezumi*	-25.815	28.407	Julius (2013)
Bronkhorstpruit, Pretoria	1	*R. rattus*	-25.805	28.746	This study
Hammanskraal scrap yard, Pretoria	1	*R. tanezumi*	-25.498	28.269	Julius (2013)
Johannesburg Zoological Gardens, Johannesburg	1	*R. norvegicus*	-26.166	28.037	Bastos *et al*. (2011)
OR Tambo International Airport, Johannesburg	1	*R. norvegicus*	-26.148	28.226	Bastos *et al*. (2011)

2.2.2 Laboratory procedures

Morphological identification of *Rattus* species was based on Brennan (1980). Three hundred and forty-two *Rattus* specimens were sexed, dissected and organ tissue (embryos and foetuses, where applicable) were removed within a biosafety level 2 (BSL2) cabinet and stored at –20^{o} C. Uteri containing small embryos and foetuses were removed from 57 pregnant *Rattus* females and placed into sterile tubes using aseptic techniques (Kosoy *et al.* 1998). Only foetuses that could be separated completely from placental tissues were used to assess the potential for vertical transmission. Foetuses removed from placental tissues and amniotic sacs were rinsed with double distilled water. In total, 87 foetuses were removed from nine *R. norvegicus* females and used for the vertical transmission screening. When removing amniotic sacs, foetuses from the same *R. norvegicus* female were separated and stored individually at –20˚ C.

Genomic DNA was extracted from 57 *Rattus* female spleen tissues and 87 foetus torsos using the High Pure PCR Template Preparation Kit (Roche Diagnostics) according to the manufacturer's protocol. The presence of *Bartonella* was tested using three primer sets, *viz*. i) Bart-EF CACGACTCYATTGATATTACAGA & *Bart-ER* GCACGTGGRTCATAATTTTTATA targeting 513 bp of the citrate synthase (*gltA*) gene region (Bastos 2007), ii) *Bart-EF* CACGACTCYATTGATATTACAGA and *BhCS1137n* AATGCAAAAAGAACAGTAAACA targeting the C-terminal 722 bp of the citrate synthase (*gltA*) gene (Birtles and Raoult 1996), and iii) *nuoG - F* GGCGTGATTGTTCTCGTTA and *nouG - R* CACGACCACGGCTATCAAT a NADH

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dehydrogenase gamma subunit (*nuoG*) gene region 346 bp in size (Colborn *et al.* 2010). PCR amplification was performed in a 50 µl reaction volume, containing 1x DreamTaq buffer (Fermentas), 0.25 µM dNTP's (Fermentas), 1U Taq polymerase (Fermentas), 100-200 ng DNA template and 0.8 µM of each primer (for primer set i), 0.5 µM of each primer (for primer set ii), and lastly, 0.4 µM of each primer (for primer set iii).

Thermal cycling followed a touchdown PCR approach as follows: primer set (ii) - initial denaturation at 96˚ C for 10 s, three cycles of denaturation at 96˚ C for 12 s, annealing at 51˚ C for 30 s and extension at 70˚ C for 70 s, five cycles of denaturation at 96˚ C for 12 s, annealing at 49.5˚ C for 25 s and extension at 70˚ C for 65 s and 35 cycles of denaturation at 96˚ C for 12 s, annealing at 48˚ C for 25 s and extension at 70˚ C for 60 s and a holding temperature at 70˚ C for 1 minute. Primer sets (i) and (iii) - initial denaturation at 96˚ C for 10 s, three cycles of denaturation at 96˚ C for 12 s, annealing at 53˚ C for 30 s and extension at 70˚ C for 55 s, five cycles of denaturation at 96˚ C for 12 s, annealing at 51.5˚ C for 30 s and extension at 70˚ C for 50 s and 35 cycles of denaturation at 96˚C for 12 s, annealing at 50˚ C for 30 s and extension at 70˚ C for 45 s and a holding temperature at 70˚ C for 1 minute.

All positive amplicons were visualised by agarose gel electorphoresis and purified directly from the tube using the Roche PCR product purification kit (Roche diagnostics) according to manufacturer's instructions. Purified PCR products were cycle sequenced using BigDye v. 3.1 terminator cycle-sequencing kit (Perkim-Elmer, Foster City, U.S.A.) using forward and reverse primers in separate reactions. Samples were run on an ABI 3130 sequencer.

2.2.3 Phylogenetic analysis

Sequence chromatograms were viewed and edited in Chromas program embedded in Mega 5 (Tamura *et al.* 2011). A BLAST nucleotide search was used (www.ncbi.nm.nih.gov/blast) to identify *Bartonella* reference species with the highest nucleotide sequence identity (> 98%) in the Genbank data base. A best-fit model of nucleotide substitution, the T92+G (G = 0.25), was identified under the Bayesian Information Criterion (BIC) in MEGA5 (Tamura *et al.* 2011). Maximum Likelihood

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(ML) and Neighbour-Joining (NJ) analyses were performed using MEGA5 (Tamura *et al.* 2011). Nodal support was assessed through 10,000 non-parametric bootstrap replications. Bayesian inference (BI) with Mr Bayes v3.1.2 (Ronquist and Huelsenbeck 2003) was performed using four chains and default heat setting and run for 5,000,000 MCMC generations.

2.3 Results

The total sample set of 342 *Rattus* samples included 186 males and 156 females. Of the latter, 57 females were pregnant (Figure 2). All pregnant females were screened for *Bartonella* presence using three primer sets. *Bartonella* genome presence was confirmed in 29 of the pregnant females by all three primer sets (*Bart-EF / ER, Bart-EF / BhCS1137n, nuoG-F / R*), in a further five pregnant females by amplification with two primer sets (*Bart-EF / ER, nuoG-F / R*), and in six additional pregnant females by repeat amplifications of a single primer set (either *Bart-EF / ER* for *nuoG - F / R*). Only nine of the 40 *Bartonella*-positive pregnant females were found to have foetuses of sufficient size to permit torso dissection. The overall prevalence of *Bartonella* in the 57 pregnant *R. norvegicus* females was high (70.2%).

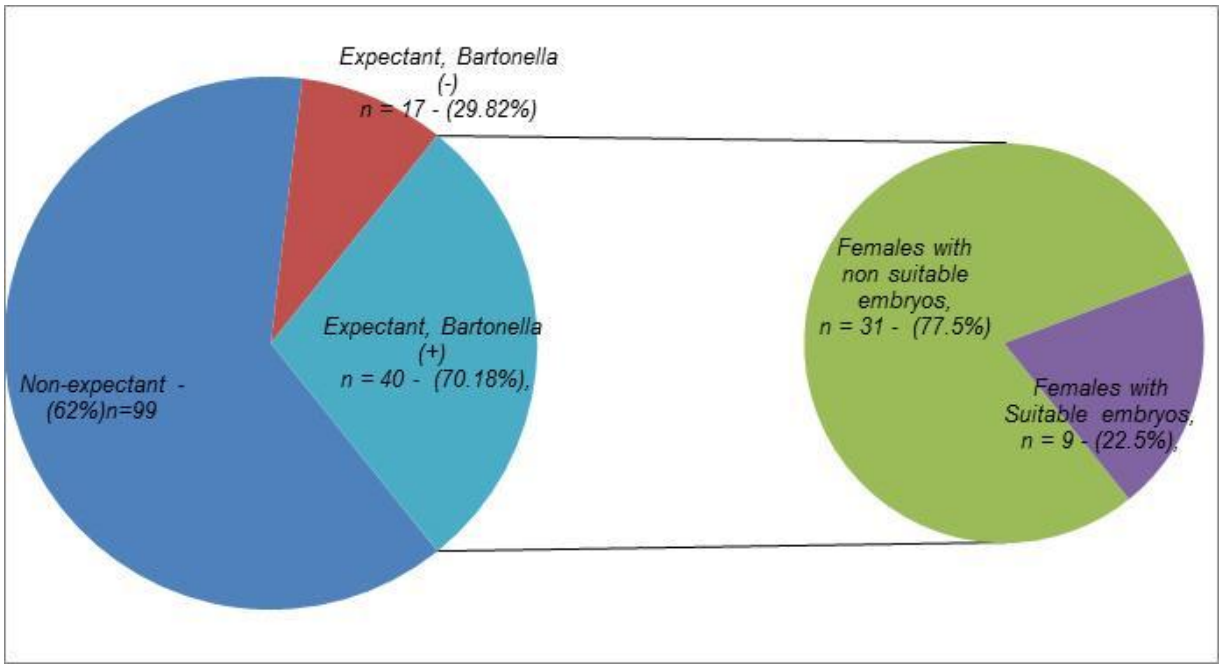

Figure 2: A summary of all *R. norvegicus* females from Gauteng Province, South Africa used to investigate vertical transmission of *Bartonella*. *Bartonella*-negatve and positive samples are represented by negative (-) and positive (+) signs, respectively.

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Screening of all 87 foetuses obtained from the nine *Bartonella*-positive mothers with the three primer sets confirmed *Bartonella* genome presence in 29 foetuses (through amplification with all three primer sets (*Bart – EF / ER, Bart – EF / BhCS1137n, nuoG – F / R*), in seven foetuses with two primer sets (B*art – EF / ER*, *nuoG – F / R*), in six foetuses with repeat amplification of primer set (*Bart – EF / ER*) and in another six foetuses with repeat amplification with primer set (*nuoG – F / R*), bringing the total number of infected foetuses to 48. The overall *Bartonella* prevalence in foetuses from infected mothers was therefore 55.2%. One PCR-positive foetus per female was randomly selected for sequencing and nucleotide blast searches confirmed that *Bartonella tribocorum* was present in four of the nine foetuses screened, and that these had 99% to 100% sequence identity to *B. tribocorum* (HG969192.1) sequences in Genbank based on the *gltA* gene region characterised (Table 2). ALX R178 foetus showed a 99% blast similarity to *Uncultured Bart RRB046N* sequence from Genbank accession number GU143527 similar to that of the mother, also based on the *gltA* gene. Foetus of ALX R166 matched *B. elizabethae* Genbank accession number EF659940.1 based on the *gltA* gene region. Feotuses from ALX134 and FR 23 where found to be *Bartonella* negative, and the JZ47 foetus produced a sequence of poor quality that could not be used with confidence in a blast search or phylogenetic analysis. Similar blast results were returned for strains present in pregnant females and their offspring.

Twelve sequences, selected from seven mothers and five foetuses, based on the partial citrate synthase gene (*gltA*) gene region from this study, were combined with 23 sequences from NCBI database with CIP isolates generated inhouse (A.D.S. Bastos unpublished) to create a homologous dataset of 35 sequences used for phylogenetic analysis. The final dataset was 475 nucleotides in length, and contained 177 variable sites, 129 parsimony-informative sites, and had a transition: transversion ratio (R) of 1.73. The average nucleotide composition was T = 31.7%, C = 21.2%, A = 27.4% and G = 19.6%.

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Table 2: *Bartonella* identified from foetuses removed from nine *Bartonella*-positive pregnant *Rattus norvegicus* females collected in Gauteng Province, South Africa between 2006 and 2013.

Pregnant females with suitable foetuses		Foetuses				
Sample number	Host Species	*Bartonella* (+) / total number of foetuses	Number of foetuses sequenced	Blast search results	Genbank Accession number	Gene region
JZ47	*R. norvegicus*	4/8	Bad seq	--	--	*NuoG*
FR23	*R. norvegicus*	0/10	0	--	--	--
ALX R43	*R. norvegicus*	11/11	1	*B. tribocorum*	HG969192.1	*gltA*
ALX R48	*R. norvegicus*	9/9	1	*B .tribocorum*	HG969192.1	*gltA*
ALX R130	*R. norvegicus*	11/11	1	*B. tribocorum*	HG969192.1	*gltA*
ALX R134	*R. norvegicus*	0/10	0	--	--	--
ALX R159	*R. norvegicus*	9/9	1	*B. tribocorum*	HG969192.1	*gltA*
ALX R166	*R. norvegicus*	1/11	1	*B. elizabethae*	EF659940.1	*nuoG*
ALX R178	*R. norvegicus*	3/8	1	*Uncultured Bart RRB046N*	GU143527.1	*gltA*

(+) = positive

Rattus norvegicus samples ALX R43, ALX R48, ALX R130, ALX R134, ALX R159 mothers and foetuses cluster with *B. tribocorum* from the Pasteur Institute's bacterial species ID number (CIP105476) with a 99% & 100% bootstrap support in the ML and N-J trees respectively, with a a BI of 100% for lineage II (Figure 3). *Rattus norvegicus* sample ALX R178 and its foetus clustered with *B. elizabethae* from the Pasteur Institute's bacterial species ID number (CIP103761) with 96% & 98% bootstrap support (ML and NJ trees, respectively) and a BI of 100% for lineage I (Figure 3).

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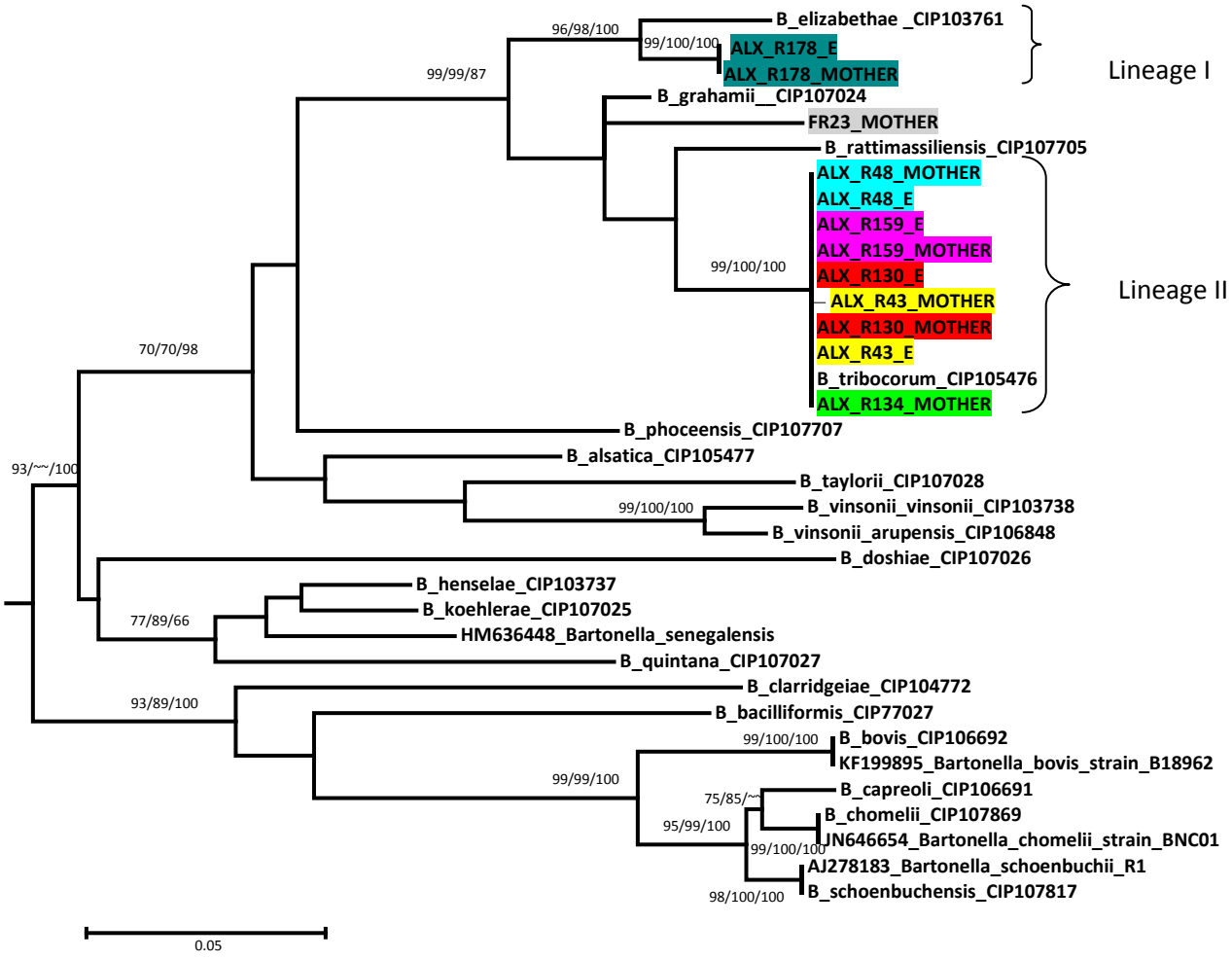

Figure 3: Maximum Likelihood (ML) tree inferred using partial *Bartonella* citrate synthase (*gltA*) gene sequences from *Rattus norvegicus* mothers and their foetuses from South Africa. Reference species names are preceded by the corresponding Genbank accession number, or where generated in-house, are followed by the CIP strain number. Nodal support values for ML and Neighbour Joining (NJ) trees are based on 10,000 non-parametric bootstrap replications and are presented together with posterior probabilities from Bayesian Inference (BI) expressed as a percentage, ML/NJ/BI on the relevant nodes.

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2.4 Discussion

This study is the first to investigate vertical transmission of *Bartonella* in invasive *R. norvegicus*. The study found a high *Bartonella* infection prevalence of 55.2% in foetuses from *Bartonella*-positive mothers. The high *Bartonella* genome prevalence in all tested foetuses confirms that vertical transmission occurs in naturally infected members of this invasive genus. This confirms previous observations made by Kosoy *et al.* (1998) of *in utero* infection of *Bartonella* in naturally infected cotton rats (*Sigmodon hispidus*) (58%) and white-footed mice (*Peromyscus leucopus*) (36.8%) with *Bartonella spp.* An experimental demonstration of transplacental transmission in bacteraemic pregnant mice was shown when 76% of resorbed mice foetuses were positive for *B. birtlesii* (Boulouis *et al*. 2001), further supporting vertical transmission of *Bartonella* in rodents.

Transmission through other maternal secretions such as milk, urine and saliva as suggested by Kosoy *et al.* (1998) could not be addressed in this study because all foetuses used were collected directly from the uterus before birth. Vertical transmission may not be the case in all mammals and may differ between species as cats that were experimentally inoculated with *B. henselae* gave birth to abacteraemic kittens and the kittens remained abacteraemic through the weaning stage (Abbott *et al.* 1997). This was also confirmed by Guptill *et al.* (1998) when kittens and foetuses from inoculated queens showed no evidence of vertical transmission of *Bartonella henselae.* The latter suggests that vertical transmission of *Bartonella* may be influenced by host and/or bacterial species.

The present study revealed high levels of *Bartonella* infection (70.2%) in pregnant *R. norvegicus* females. Although this prevalence was not compared to that of non-pregnant *R. norvegicus* females since non-pregnant females were not screened for *Bartonella* presence in this study, we do know from a previous study that *Bartonella* prevalence in invasive *Rattus* from South Africa was higher *R. norvegicus* females (32.6%) compared to males (20.4%; Brettschneider 2011). The high levels of *Bartonella* infection in pregnant females found in this study, provides a plausible explanation for the higher rates of infection detected previously. A number of earlier findings, including those of Bouloius *et al.* (2001) found higher levels of *B.*

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birtlesii in pregnant mice than in non-pregnant mice. A study in France also showed significantly higher infection levels of *B. bovis* in pregnant cows than in non-pregnant cows, with the level of bacteraemia increasing in the last two-thirds of the gestation period (Maillard *et al.* 2006). It was suggested that the reason for high bacteremia in these pregnant mammals may be due to hormonal changes during pregnancy (Bloulois *et al.* 2001).

The high *Bartonella* prevalence in pregnant mammals may pose a potential problem for livestock and human populations. Experimental transplacental transmission of *B. birtlesii* in mice showed an increase in foetal deaths, increased foetal resorption and low birth weight (Bloulois *et al*. 2001). Infection of female cats inoculated with *B. henselae* was accompanied by decreased conception and failure to maintain pregnancy (Guptill *et al.* 1998). Furthermore, *B. henselae* was also detected in an aborted equine foetus, supporting suggestions that members of this bacterial genus may be responsible for reproductive disorders (Johnson *et al.* 2009).

Structural differences in placentation could be the cause for mother to offspring transmission of the bacteria (Breitschwerdt *et al.* 2010). The murine model of brucellosis, which is also a member of alpha proteobacteria, suggests that the placenta may be the site for *Brucella* multiplication which could also be the case for *Bartonella* (Watanabe *et al*. 2008). Boulouis *et al.* (2001) showed that there was evidence of vascular lesions on placentas of non-resorbed *Bartonella*-positive foetuses. Efforts to amplify *Bartonella spp.* DNA from a *Bartonella*-positive mother's paraffin-embedded placental tissue in humans were not successful, suggesting that caesarean section and blood transfusion may be another mechanism for *Bartonella* transmission to offspring (Breitswerdt *et al.* 2010).

Bartonella sequences generated in this study clustered with *B. elizabethae* and *B. tribocorum* which are known rodent-borne zoonotic pathogens, causing endocarditis and febrile illness in humans, respectively (Buffet *et al*. 2013). Understanding *Bartonella* transmission in invasive *Rattus* species will contribute to our understanding of their infection dynamics. Brettschneider *et al*. (2012) developed a mathematical model to study the dynamics of *Bartonella* infections in invasive

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commensal *Rattus* species from South Africa, but this model was constrained by the limited data available for a number of key biological parameters. The 55.2% *Bartonella* infection prevalence confirming vertical transmission was detected in this study and provides a valuable empirical estimate for a key biological parameter that will improve future revisions of this mathematical model.

Samples used in this study were collected in semi-urban and urban informal settlements of Gauteng Province, South Africa. This close association of *Rattus* with humans poses a zoonotic threat. A great percentage of *Rattus* specimens collected in this study are from informal settlements where opportunistic bacterial infections seem to thrive in immunocompromised individuals (Koehler *et al.* 1995) and lack of adequate infrastructure increases their vulnerablility and susceptibility to these infections, including *Bartonella*.

This study is the first to provide molecular evidence that vertical transmission is one of the mechanisms used by the genus *Bartonella* to maintain infection in South African invasive *Rattus* species populations and provides new data that may assist medical, veterinary, municipal, conservation and agricultural authorities in formulating strategies to manage problems associated with these rodent pests.

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Chapter 3

Prevalence of two zoonotic pathogens, *Bartonella elizabethae* and *B. tribocorum* detected in *Rattus*-associated ectoparasites in Gauteng Province, South Africa

Abstract

Vector transmission of *Bartonella* is well-established with *Bartonella* known to be transmitted through bites and contact with feaces of infected ticks, lice, mites and fleas. The present study investigated *Bartonella* genome presence in *Rattus*-associated ectoparasites from invasive *Rattus* species sampled in Gauteng Province, South Africa by targeting the *gltA* and *nuoG* gene regions. A total of 1400 ectoparasites were collected, these included fleas (*n* = 112), ticks (*n* = 25), mites (*n* = 392), and lice (*n* = 871). *Bartonella* genome presence was detected in fleas (*Xenopsylla brasiliensis*, and *X. cheopis)* and in *Haemaphysalis elliptica* ticks at prevalences of 84.3% and 0%, repectively. In contrast, the *Rattus* host prevalence was intermediate, at 54.1% and approximately 50% of the *Bartonella* strains detected in the vertebrate host coincided with the *Bartonella* strains identified in the corresponding flea vector. These results indicate that fleas play an important role in vectoring *Bartonella*. The remaining 50% of *Bartonella* strains identified in *Rattus* hosts were found to differ from the *Bartonella* variants identified in their corresponding fleas suggesting that fleas feed on multiple hosts infected with different *Bartonella* species. Phylogenetic analysis showed that the *Bartonella* flea and host sequences clustered within two lineages (denoted II and III) corresponding to *B. elizabethae* and within lineage I clustering with *B. tribocorum*. These findings support the results of the mathematical model which indicated that fleas likely play a key role in the epidemiology of *Bartonella* in invasive rats. The high levels of infection of the rats and their ectoparasites, with two zoonotic *Bartonella* species, *B.* elizabethae and *B. tribocorum*, are of public health concern.

Keywords: *Rattus, Bartonella*, ectoparasites, *Xenopsylla brasiliensis*, *X. cheopis, Haemaphysalis elliptica,* vector transmission, *gltA*, *nuoG*, South Africa.

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3.1 Introduction

3.1.1 Vector-borne diseases

Some of the world's most destructive diseases are vector-borne, contributing to society's burden of morbidity and mortality (Spellman and Stoudt 2013). These infectious diseases are not easily controlled or prevented if the vector's biology, population dynamics and transmission routes are not well-understood. Haematophagous arthropods are known to be responsible for transmission of many vertebrate diseases caused by viruses, bacteria, protozoa and helminths (Gubler *et al.* 1991). Arthropod ectoparasites are a diverse and highly adapted group of organisms that can occupy the host's nest or may sometimes live permanently on their host (Hanafi-bojd *et al*. 2007).

Examples of major vector-borne diseases include: Lyme disease caused by *Borrelia burgdorferi*, transmitted to humans through an infected tick bite (Steere *et al.* 2004); plague, caused by *Yersinia pestis*, transmitted by an infected flea (Chain *et al.* 2004); Chikungunya fever, caused by an arthropod-borne alphavirus, Chikungunya virus, transmitted by infected mosquitos (Staples *et al.* 2009); malaria caused by members of the genus *Plasmodium*, transmitted by infected mosquitos (Miller *et al.* 2002; Foller *et al.* 2009); Dengue fever, caused by dengue virus, transmitted by infected mosquitos (Schul *et al.* 2007); yellow fever caused by a single-stranded positive-sense RNA yellow fever virus of the genus *Flavivirus*, transmitted by infected mosquitos (Bryant *et al.* 2007); Leishmaniasis, a tropical disease caused by different species of protozoan parasite of the genus *Leishmania*, transmitted by sandflies; the deadly louse-borne relapsing fever caused by the tick-borne *Borrelia recurrentis*; and tick-borne relapsing fever caused by *Borrelia duttoni* (Gubler 1998; Lescot *et al*. 2008; Spellman and Stoudt 2013).

3.1.2 Bartonellosis and rodents

Bartonellosis is considered to be a re-emerging vector-borne disease caused by aerobic, fastidious, gram-negative bacteria, all features inherent in the alpha-2 subgroup of the class proteobacteria (Bown *et al.* 2004; Chomel *et al.* 2006; Bitman *et al* 2010; Bitman *et al.* 2012). Currently, the genus *Bartonella* consists of 34 recognized species that parasitize endothelial and erythrocyte cells of mammalian

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hosts, and which are increasingly implicated in a number of diseases (Schulein *et al.* 2001; Bitman *et al.* 2012; Eicher *et al.* 2012; Buffet *et al.* 2013; Rasis et al. 2014). Members of the genus *Bartonella* are known to cause Carrion's disease, Oroya fever, trench fever, cat-scratch diseases, febrile illness, neuroretinitis, endocarditis, myocarditis, lymphadenitis and hepatitis (Townsend *et al.* 1913; Vinson *et al.* 1969; Kerkhoff *et al.* 1999; Daly *et al.* 1993; Agan and Dolan 2002; Kosoy *et al.* 2003; Billeter *et al.* 2008; Kosoy *et al.* 2010; Kaiser *et al.* 2011). Twenty of the currently recognized species of *Bartonella* have been detected in rodents (Buffet *et al.* 2013) with *B. coopersplainsensis*, *B. elizabethae*, *B. grahamii*, *B. phoceensis*, *B. queenslandensis*, *B. rattaustraliani*, *B. rattimassiliensis*, *B. rochalimae*, and *B. tribocorum* isolated from *Rattus* species. (Daly *et al*. 1993; Birtles *et al.* 1995; Heller *et al.* 1998; Gundi *et al.* 2004; Gundi *et al.* 2009; Lin *et al.* 2008).

Members of the genus *Rattus* are a successful invasive commensal group of mammals that have proved to be a nuisance to humankind (Chinen *et al*. 2005; Davis *et al.* 2005). An increase in human population in urban environments has been reported to go hand-in-hand with increases in *Rattus* populations (Himsworth *et al.* 2013; 2014; Kajdacsi *et al.* 2013) because cities provide good harbourage and food resources for rats. The human population increase in cities and the expansion of informal settlements, epitomised by poor housing and sewerage systems and uncontrolled garbage disposal, forms an ideal environment for members of the genus *Rattus* to flourish (Taylor *et al.* 2008). This poses a public health risk considering that members of the genus *Rattus* are known to be infested with a diverse range of ectoparasites such as ticks and mites, and to harbour a broad range of zoonotic pathogens, including hepatitis E virus, *Leptospira interrogans*, *Bartonella*, *Rickettsia typhi*, Seoul virus and endoparasites such as *Calduim hepatica* and *Hymenolepis sp*. (Earsterbrook *et al.* 2007). Three invasive commensal *Rattus* species have been recorded to occur in South Africa and include *R. rattus, R. norvegicus and R. tanezumi* (Bastos *et al.* 2011).

3.1.3 Arthropod vectors

*Bartonell*a has not only been identified in *Rattus* species, but also in a number of mammalian arthropod ectoparasites such as fleas, lice, sand flies, ticks, biting

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flies, keds (or louse flies) and mites (Townsend *et al.* 1913; Noguchi *et al.* 1929; Chomel *et al.* 1996; Finkelstein *et al.* 2002; Fournier *et al.* 2002; Halos *et al.* 2004; Reeves *et al.* 2006; Matsumoto *et al.* 2008; Angelakis *et al.* 2010a; Mediannikov *et al.* 2014). However, *Bartonella* vector competencies have not been demonstrated for many of these ectoparasites, with the exception of some species of fleas, lice, sand flies and ticks (Chomel *et al.* 2009). Experimental studies are needed to demonstrate vector competencies or reliable transmission between vector and host before declaring an arthropod to be a vector for any disease (Billeter *et al.* 2008).

3.1.4 Ticks

Noguchi (1926) was the first to demonstrate the role of ticks in *Bartonella* infections, and many subsequent studies showed the potential for ticks to be vectors of *Bartonella* (Chang *et al.* 2002; Skotarczak and Adamska 2005; Holden *et al.* 2006; Winkswo *et al*. 2007; Angelakis *et al.* 2010a; Mediannikov *et al.* 2014). However, it was only relatively recently when Cotte *et al.* (2008) demonstrated successful experimental transmission of *B. henselae* from the salivary glands of ticks (*Ixodes ricinus*) to cats that the vector competency of ticks in *Bartonella* infections was proven.

3.1.5 Lice and sandflies

In contrast to ticks, a number of experiments were conducted demonstrating lice to be competent vectors for *B. quintana*, the causative agent for trench fever (Bruce *et al.* 1921; Mooser and Weyer 1953; Vinson *et al.* 1969; Raoult and Roux 1999; Fournier *et al.* 2001). Lice transmit *Bartonella* mainly through feces, where the defecated feces causes a local irritation that results in itching and scratching, facilitating inoculation of the infected feces into the host through broken skin (Raoult and Roux 1999). Studies investigating *Bartonella* prevalence in different lice species and in homeless people were conducted to identify ways to curb *Bartonella* transmission causing trench fever (Reeves *et al.* 2006; Sasakie *et al.* 2006; Alcantara *et al.* 2009; Bonilla *et al.* 2009).

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Sandflies were first identified as the natural vectors of *B. bacilliformis* causing Verruga peruana (Townsend 1913). Subsequent transmission experiments with wild sandflies proved vector competence of sandflies in *Bartonella* transmission (Hertig 1942a, b).

3.1.6 Fleas

Brettschneider *et al*. (2012) predicted that fleas are primary vectors of *Bartonella* transmission in *Rattus*, based on a mathematical model developed to investigate if differential ectoparasite load *in R. rattus* and *R. norvegicus* from South Africa fully explains the difference in *Bartonella* infection prevalence in these two *Rattus* species. Historically, Koehler *et al.* (1994) was the first study to isolate *B. henselae* from cat fleas (*Ctenocephalides felis*) in bacteraemic cats in northern California, U.S.A. and Chomel *et al.* (1996) showed that the cat flea was the main vector of *B. henselae*, an agent of cat scratch disease. Subsequently, several studies demonstrated the role of fleas as competent vectors of *Bartonella* transmission (Higgins *et al.* 1996; Foil *et al*. 1998; Finkelstein *et al.* 2002; Engel and Dehio 2009).

Bartonella species that include *B. clarridgeiae*, *B. koehlerae, B. vinsonii subsp. berkhoffii*, *B. rochalimae*, *B. quintana*, *B. taylorii*, *B. doshiae*, *B. elizabethae*, *B. grahamii*, *B. vinsonii subsp. vinsonii*, *B. tribocorum*, *B. queenslandensis*, *B. alsatica candidatus* and *B. antechini* have previously been isolated from confirmed vectors that include mammalian flea species using culture and molecular techniques (Tsai *et al.* 2011). The ability of fleas to harbour and transmit such a wide range of *Bartonella* species is not fully understood, however fleas are not host-specific and some flea species (e.g., rabbit fleas) are able to survive in harsh environments by entering diapause as fed adults (Telfer *et al.* 2007). Higgins *et al.* (1996) showed that *B. henselae* can multiply in the digestive system of cat fleas when *Bartonella* was found to be present in the flea gut three hours post-feeding and persisted up to nine days after infection. The *Bartonella* species detected in the flea may not necessarily be the same as the *Bartonella* detected in the flea host (Brinkerhoff *et al.* 2010). This may be because fleas take blood meals from multiple host species. Additionally, co-infection of *Bartonella* in rodent fleas was also reported by Abbot *et al.* (2007)

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suggesting that *Bartonella* infection in fleas may not automatically mean that the host is infected with the same *Bartonella* species (Brinkerhoff *et al.* 2010). It is suggested that the transmission of *Bartonella* is through inoculation of flea feces by a cat scratch (*Bartonella* can survive in flea feces for several days) or through a flea bite (Chomel *et al.* 1996; Foil *et al.*1998).

The aim of the present study is to identify arthropod ectoparasites infesting *Rattus* species in urban (informal settlements in the city centre) and peri-urban (settlements on the city outskirts) areas of Gauteng Province, South Africa. The focus on urban and peri-urban sampling sites in this study was primarily due to the high *Bartonella* prevalence previously found in *Rattus* from urban (19%) and peri-urban (17%) sampling sites in South Africa (Brettschneider 2011). The study also investigated the prevalence and diversity of *Bartonella* in *Rattus*-associated ectoparasites in order to assess vector potential of the ectoparasites.

3.2 Material and methods

3.2.1 Sample collection

Rattus samples (*R. norvegicus* and *R. tanezumi*) used in the present study were collected between 2010 and 2011 (Julius 2013) and between 2012 and 2013 (this study). *Rattus rattus* infested with ticks and fleas were not sampled during either of these periods; hence this species did not form part of the present study. All *Rattus* specimens infested with ticks and fleas are shown in Table 2. Sampling was targeted at four localities in Gauteng Province, South Africa (Figure 1; Table 1). Between 2012 and 2013 *Rattus* specimens were snap-trapped, live-trapped by members of the public during an extermination program conducted by Johannesburg Municipality, or obtained through donations from an Agriculture Research Council (ARC) research project, Rietondale campus Pretoria. Live-trapped *Rattus* specimens were baited with a mixture of peanut butter, oats and canned fish and were sacrificed using CO_2 and immediately placed in individual bags. The samples were collected under collection permit CPF6 0032 issued by the Department of Nature Conservation in Gauteng and Animal ethics permit ECO25-10 approved by the Animal Ethics Committee of University of Pretoria, South Africa. Permission to

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conduct research in terms of section 20 of the Animal Disease Act (Act No 35 of 1984) was granted by the Department of Agriculture, Forestry and Fisheries (Ref 13788) (Appendix 1). All *Rattus* specimens infested with ticks and fleas are shown in Table 2.

Table 1: Geographic coordinates of localities where host species (*Rattus* spp) were sampled in Gauteng Province, South Africa.

Locality	n	Species name	latitude	longitude	Reference
Alexandra	53	*R. norvegicus*	-26.623	28.577	This study
Bronkorstpruit, Pretoria	3	*R. norvegicus*	-25.805	28.746	This study
Hammanskraal scrap yard	5	*R. tanezumi*	-25.404	28.263	Julius (2013)
Tembisa	13	*R. norvegicus*	-26.166	28.037	Julius (2013)

Table 2: *Rattus* host species and their associated ectoparasites sampled in Gauteng Province, South Africa.

Sample number	Host species name	Host's gender	No. of ectoparasites per rat	Ectoparasite species name	Ectoparase gender
ALX-R2	*R. norvegicus*	F	1	*X. brasiliensis*	F
ALX-R06	*R. norvegicus*	M	1	*X. brasiliensis*	F
ALX-R07	*R. norvegicus*	F	1	*X. brasiliensis*	F
ALX-R09	*R. norvegicus*	F	1	*X. brasiliensis*	M
ALX-R14	*R. norvegicus*	M	1	*X. brasiliensis*	M
ALX-R19	*R. norvegicus*	F	1	*X. brasiliensis*	M
ALX-R22	*R. norvegicus*	F	1	*X. brasiliensis*	F
ALX-R26	*R. norvegicus*	M	2	*X. brasiliensis*	F
				X. brasiliensis	F
ALX-R27	*R. norvegicus*	M	2	*X. brasiliensis*	M
				X. brasiliensis	F
ALX-R32	*R. norvegicus*	M	2	*X. brasiliensis*	M
				X. brasiliensis	F
ALX-R35	*R. norvegicus*	M	1	*X. brasiliensis*	F
ALX-R37	*R. norvegicus*	F	3	*X. brasiliensis*	M1
				X. brasiliensis	M2
				X. brasiliensis	M3
ALX-R42	*R. norvegicus*	F	2	*X. brasiliensis*	M1
				X. brasiliensis	M2
ALX-R49	*R. norvegicus*	F	1	*X. brasiliensis*	F
ALX-R50	*R. norvegicus*	F	2	*X. brasiliensis*	M1
				X. brasiliensis	M2

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Sample number	Host species name	Host's gender	No. of ectoparasites per rat	Ectoparasite species name	Ectoparase gender
ALX-R63	*R. norvegicus*	M	1	*X. brasiliensis*	F
ALX-R74	*R. norvegicus*	M	4	*X. brasiliensis*	M1
				X. brasiliensis	M2
				X. brasiliensis	M3
				X. brasiliensis	F
ALX-R78	*R. norvegicus*	M	1	*X. brasiliensis*	M
ALX-R81	*R. norvegicus*	F	1	*X. brasiliensis*	M
ALX-R83	*R. norvegicus*	M	1	*X. Cheopis*	F
ALX-R92	*R. norvegicus*	F	3	*X. brasiliensis*	M1
				X. brasiliensis	M2
				X. brasiliensis	F
ALX-R93	*R. norvegicus*	M	2	*X. brasiliensis*	M
				X. brasiliensis	F
ALX-R98	*R. norvegicus*	M	1	*X. brasiliensis*	F
ALX-R100	*R. norvegicus*	M	3	*X. brasiliensis*	M
				X. brasiliensis	F
				X. brasiliensis	F
ALX-R105	*R. norvegicus*	M	1	*X. brasiliensis*	F
ALX-R107	*R. norvegicus*	M	3	*X. brasiliensis*	M1
				X. brasiliensis	M2
				X. brasiliensis	M3
ALX-R110	*R. norvegicus*	M	1	*X. Cheopis*	F
ALX-R126	*R. norvegicus*	F	3	*X. brasiliensis*	M
				X. brasiliensis	F1
				X. brasiliensis	F2
ALX-R127	*R. norvegicus*	M	2	*X. brasiliensis*	M
				X. brasiliensis	F
ALX-R128	*R. norvegicus*	M	4	*X. brasiliensis*	M1
				X. brasiliensis	M2
				X. brasiliensis	F
				X. brasiliensis	M
ALX-R129	*R. norvegicus*	F	1	*X. brasiliensis*	F
ALX-R132	*R. norvegicus*	F	1	*X. brasiliensis*	M
ALX-R133	*R. norvegicus*	F	1	*X. brasiliensis*	F
ALX-R148	*R. norvegicus*	M	1	*X. brasiliensis*	M
ALX-R152	*R. norvegicus*	M	1	*X. brasiliensis*	M
ALX-R153	*R. norvegicus*	F	1	*X. brasiliensis*	F
ALX-R166	*R. norvegicus*	F	1	*X. brasiliensis*	M
ALX-R168	*R. norvegicus*	F	1	*X. brasiliensis*	M
ALX-R170	*R. norvegicus*	M	4	*X. brasiliensis*	M
				X. brasiliensis	F1
				X. brasiliensis	F2
				X. brasiliensis	F

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Sample number	Host species name	Host's gender	No. of ectoparasites per rat	Ectoparasite species name	Ectoparase gender
ALX-R172	*R. norvegicus*	M	1	*X. brasiliensis*	F
ALX-R174	*R. norvegicus*	M	1	*X. brasiliensis*	M
ALX-R178	*R. norvegicus*	F	3	*X. brasiliensis*	M1
				X. brasiliensis	M2
				X. brasiliensis	F
ALX-R179	*R. norvegicus*	M	4	*X. brasiliensis*	M1
				X. brasiliensis	M2
				X. brasiliensis	M3
				X. brasiliensis	F
ALX-R185	*R. norvegicus*	F	3	*X. brasiliensis*	M
				X. brasiliensis	F
				X. brasiliensis	F
ALX-R202	*R. norvegicus*	F	2	*X. brasiliensis*	M
				X. brasiliensis	F
ALX-R203	*R. norvegicus*	M	3	*X. brasiliensis*	M1
				X. brasiliensis	M2
				X. Cheopis	F
ALX-R204	*R. norvegicus*	F	1	*X. brasiliensis*	M
ALX-R212	*R. norvegicus*	M	1	*X. brasiliensis*	M
ALX-R216	*R. norvegicus*	F	1	*X. brasiliensis*	F
ALX-R219	*R. norvegicus*	F	2	*X. brasiliensis*	M1
				X. brasiliensis	M2
ALX-R230	*R. norvegicus*	M	2	*X. brasiliensis*	M
				X. brasiliensis	F
ALX-R268	*R. norvegicus*	M	1	*X. brasiliensis*	M
ALX-R281	*R. norvegicus*	M	1	*X. brasiliensis*	F
TEM-R31	*R. norvegicus*	M	1	*X. brasiliensis*	F
TEM-R53	*R. norvegicus*	F	4	*X. brasiliensis*	M1
				X. brasiliensis	M2
				X. brasiliensis	M3
				X. brasiliensis	F
TEM-R45	*R. norvegicus*	F	4	*X. brasiliensis*	M
				X. brasiliensis	F1
				X. brasiliensis	F2
				X. brasiliensis	F3
			1	*H. elliptica*	larvae
TEM-R43	*R. norvegicus*	M	1	*X. brasiliensis*	M
			1	*H. elliptica*	larvae
TEM-R27	*R. norvegicus*	M	2	*X. brasiliensis*	F1
				X. brasiliensis	F2
TEM-R52	*R. norvegicus*	F	1	*X. brasiliensis*	M
TEM-R55	*R. norvegicus*	M	3	*X. brasiliensis*	M1
				X. brasiliensis	M2

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Sample number	Host species name	Host's gender	No. of ectoparasites per rat	Ectoparasite species name	Ectoparase gender
				X. brasiliensis	F
TEM-R48	*R. norvegicus*	F	1	*X. brasiliensis*	F
			1	*H. elliptica*	M
TEM-R47	*R. norvegicus*	F	2	*X. brasiliensis*	M1
				X. brasiliensis	M2
TEM-R49	*R. norvegicus*	F	1	*X. brasiliensis*	F
			1	*H. elliptica*	larvae
TEM-R51	*R. norvegicus*	F	1	*X. brasiliensis*	F
TEM-R80	*R. norvegicus*		1	*H. elliptica*	M
TEM-R73	*R. norvegicus*		4	*H. elliptica*	larvae
				H. elliptica	larvae
				H. elliptica	larvae
				H. elliptica	nymph
HUP-R58	*R. tanezumi*	F	1	*H. elliptica*	larvae
HUP-R56	*R. tanezumi*	F	5	*H. elliptica*	larvae
				H. elliptica	larvae
				H. elliptica	larvae
				H. elliptica	larvae
		M		*H. elliptica*	larvae
HUP-R82	*R. tanezumi*		2	*H. elliptica*	larvae
				H. elliptica	larvae
HUP-R34	*R. tanezumi*	F	1	*H. elliptica*	larvae
HUP-R35	*R. tanezumi*	F	4	*H. elliptica*	F
				H. elliptica	larvae
				H. elliptica	larvae
				H. elliptica	larvae
FR27	*R. norvegicus*		1	*H. elliptica*	larvae
FR49	*R. norvegicus*		1	*H. elliptica*	F
FR74	*R. norvegicus*		1	*H. elliptica*	larvae

F = Female, M = Male, ALX = Alexandra, TEM = Tembisa, HUP = Hammanskraal, University of Pretoria Campus, FR = Bronkhorstspruit.

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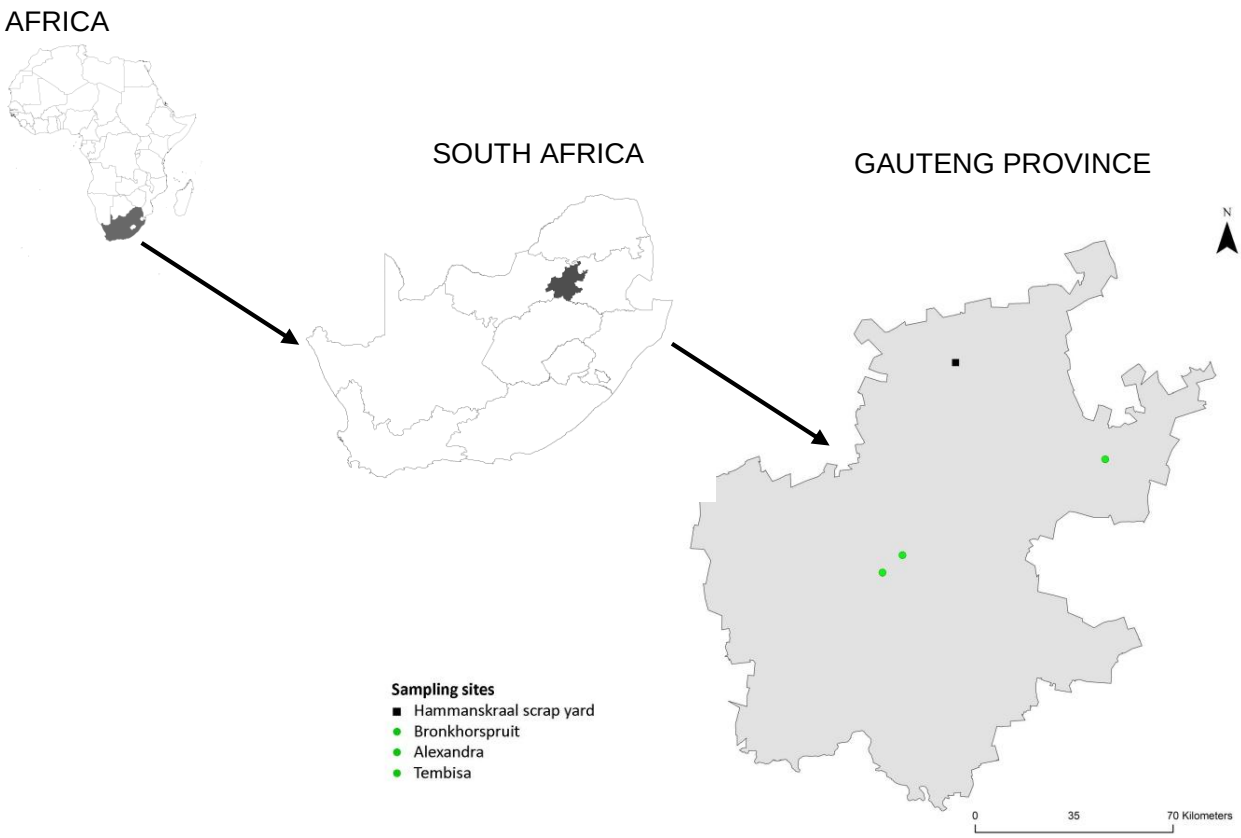

Figure 1: Map of Gauteng Province indicating the four sampling sites for *Rattus* spp. sampled between 2011 and 2012.The green sampling points are areas where *R. norvegicus* was sampled, and the black point corresponds to the area where *R. tanezumi* was sampled.

3.2.2 Laboratory procedures

A total of 1400 ectoparasites were individually removed by combing the fur of 450 *Rattus* specimens in a biological safety hood. All ectoparasites collected were preserved in 100% ethanol and subsequently sorted into mites, ticks, lice and fleas for further identification. Ectoparasites were identified to the species level using published morphological identification keys for fleas (Ewing 1943; Traub 1950; De Meillon *et al.* 1968; Segerman 1995) and ticks (Hoogstraal 1958, 1964; Hoogstraal and Kammah 1972; Pegram *et al.* 1981; Camicas *et al.* 1998; Apanaskevich *et al.* 2007). Mites were identified to the genus level, and where possible to the species level using published morphological identification keys (Pratt 1963; Strandtmann and Mitchell 1963; Bakker 1999) and through the assistance of an Acarologist, Dr Eddie Ueckermann of the Department of Entomology, Agriculture Research Council (ARC), Pretoria, South Africa.

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Whole ectoparasites were individually crushed using sterile sand and a pestle, and eluted in 200 µl of phosphate buffered saline (PBS). Genomic DNA was extracted from host spleen tissues (*n* = 74) and from ectoparasite homogenates of fleas (*n* = 108) and ticks (*n* = 25) using the High Pure PCR Template Preparation Kit (Roche Diagnostics), according to the manufacturer's protocol, with the exception that ectoparasite DNA was eluted in half the advocated volume (100 µl instead of 200 µl). All extracts were stored at -20˚ C until further processing.

Bartonella genome presence was evaluated using three primer sets, viz. i) Bart - EF CACGACTCYATTGATATTACAGA & Bart - ER GCACGTGGRTCATAATTTTTATA targeting 513 bp of the citrate synthase (*gltA*) gene region (Bastos 2007), ii) Bart - EF CACGACTCYATTGATATTACAGA and BhCS1137n AATGCAAAAAGAACAGTAAACA targeting 722 bp of the citrate synthase (*gltA*) gene region (Birtles and Raoult 1996), and iii) nuoG - F GGCGTGATTGTTCTCGTTA and nouG - R CACGACCACGGCTATCAAT of the NADH dehydrogenase gamma subunit (*nuoG*) gene region 346 bp (Colborn *et al.* 2010). PCR amplification was performed in a 50 µl reaction volume, containing 1x buffer (Fermentas), 0.25 µM dNTP's (Fermentas), 1U Taq polymerase (Fermentas), 100-200 ng DNA template and 0.8 µM of each primer for primer set (i), 0.5 µM for primer set, (ii) and 0.4 µM for primer set (iii).

Thermal cycling was performed using a touchdown PCR approach. For primer set (ii) - initial denaturation at 96˚ C for 10 s, three cycles of denaturation at 96˚ C for 12 s, annealing at 51˚ C for 30 s and extension at 70˚ C for 70 s, five cycles of denaturation at 96˚ C for 12 s, annealing at 49.5˚ C for 25 s and extension at 70˚ C for 65 s and 35 cycles of denaturation at 96˚ C for 12 s, annealing at 48 for 25 s and extension at 70˚ C for 60 s and a final extension step at 70˚ C for 1 minute. For primer sets (i) and (iii) initial denaturation at 96˚ C for 10 s, three cycles of denaturation at 96˚ C for 12 s, annealing at 53˚ C for 30 s and extension at 70˚ C for 55 s, five cycles of denaturation at 96˚ C for 12 s, annealing at 51.5˚ C for 30 s and extension at 70˚ C for 50 s and 35 cycles of denaturation at 96˚ C for 12 s, annealing at 50˚ C for 30 s and extension at 70 ˚C for 45 s and a final extension step at 70˚ C for 1 minute.

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Roche PCR product purifications (Roche Diagnostics) were performed according to manufacturer's instructions, with at least one of the three amplicons being sequenced for each positive sample. Purified PCR products were cycle sequenced using BigDye v. 3.1 terminator cycle-sequencing kit (Perkim-Elmer, Foster City, and U.S.A.) with each of the forward and reverse primers, in separate reactions. Reactions were denatured and run on an ABI 3130 sequencer.

3.2.3 Phylogenetic analysis

Sequence chromatograms were viewed and edited in the Chromas program embedded in MEGA5 (Tamura *et al.* 2011). A BLAST nucleotide search was used (www.ncbi.nm.nih.gov/blast) to identify *Bartonella* reference species with the highest nucleotide sequence identity (> 99%) in the Genbank database. A best-fit substitution model T92+G (G=0.25) was selected under the Bayesian Information Criterion (BIC) in Mega5 (Tamura *et al.* 2011). Maximum Likelihood (ML) and Neighbour-Joining (NJ) trees were inferred in MEGA5 (Tamura *et al.* 2011) using this model of sequence evolution and nodal support was assessed through 10,000 non-parametric bootstrap replications. The Bayesian Inference (BI) was run to 5,000,000 MCMC generations in Mr Bayes v3.1.2 (Ronquist and Huelsenbeck 2003) and 25% of the run was discarded as burn-in.

3.3 Results

A total of 1400 ectoparasites were collected from 450 *Rattus spp.* sampled from urban and peri-urban areas of Gauteng Province, South Africa (Tables 1 and 2). These include fleas (*n* = 112), ticks (*n* = 25), mites (*n* = 392), and lice (*n* = 871). The different ectoparasites were identified to genus level and where possible species level and included *Xenopsylla brasiliensis* and *X. cheopis* fleas, *Haemaphysalis elliptica* ticks and *Ornithonyssuss bacoti* and *Laelaps spp* mites.

All 25 ticks were screened for *Bartonella* presence using the three primer sets. There was no evidence of *Bartonella* infection in ticks. Four fleas were retained as reference samples for identification purposes and the remaining 108 fleas were screened for *Bartonella* genome presence using all three primer sets. *Bartonella* was identified in 91 (84.3%) of the fleas screened, with presence in 78 fleas being

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confirmed through amplification with all three primer sets (*Bart – EF / ER*, *Bart – EF / BhCS1137n*, *nuoG – F / R*). A further two fleas were identified as positive through repeat amplifications for primer set *Bart-EF /E R*, six fleas with repeat amplifications for primer set *Bart - EF / ER* and *Bart – EF / BhCS1137n*, two fleas with amplification for *Bart – EF / ER* and *nuoG – F / R*, two fleas with amplification for primer set *Bart – EF / BhCS1137n* and *nuoG – F / R* and lastly one flea was scored positive following repeat amplifications with the *Bart – EF / BhCS1137n* primer set.

From the 450 *Rattus spp* sampled, 74 (*R. tanezumi* - *n* = 5 and *R. norvegicus* - *n* = 69) were coinfested with ticks and fleas and screened for *Bartonella* genome presence. *Bartonella* was identified in 40 of the 74 individuals (54.1%) that were screened, with 23 *R. norvegicus* samples being identified as positive through amplification with all three primer sets (*Bart – EF / ER, Bart – EF / BhCS1137n, nuoG – F / R*), 11 *R norvegicus* and one *R. tanezumi* scored positive based on *Bart – EF / ER* and *nuoG – F / R* amplification, three *R. norvegicus* individuals confirmed positive through repeat amplifications with primer *set Bart – EF / ER*, and lastly an additional two *R. norvegicus* samples being scored as positive based on repeat amplifications with primer set (*nuoG – F / R*).

The *gltA* gene region of a subset of 22 positive fleas (11 male and 11 female fleas), randomly selected from the 91 *Bartonella*-positive fleas, and corresponding to 24.2% of the total, was sequenced. Nine of the sequenced fleas showed > 99% blast similarity to *B. tribocorum* sequence from Genbank accession number HG969192.1. Six sequences showed 100% blast similarity to an uncultured *Bartonella sp.* clone EZ8.6 Genbank accession number HM749297.1 previously identified from a Namaqua rock mouse, *Michaelamys namaquensis* sampled from Ezemvelo Nature Reserve, Gauteng Province, South Africa (Brettschneider *et al.* 2012). Four sequences showed a 100% blast similarity to an uncultured *Bartonella sp*. clone ARC131 Genbank accession number JQ352095.1 detected in a *R. tanezumi* individual collected from Tholakeke, Gauteng Province, South Africa (Mostert 2009). The remaining three flea sequences showed a 100% blast similarity to uncultured *Bartonella sp.* clone OT34 Genbank accession number JQ352089.1 identified in a *R. norvegicus* indivudual sampled from O.R. Tambo International Airport, Gauteng Province, South Africa (Brettschneider 2011).

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The 22 sequenced fleas randomly selected for genetic characterisation were collected from 19 [*Bartonella* positive (n=10) and (n=9) *Bartonella* negative] of the corresponding 74 *Rattus* hosts corresponding to 25.7% of the total number of hosts determined to be coinfested with ticks and fleas. Ten of the corresponding 40 *R. norvegicus* hosts (25%) found to be *Bartonella*-positive were selected for genetic characterisation. Nucleotide sequencing of the *gltA* gene region from the positive hosts revealed that four *R. norvegicus* individuals harboured *B. tribocorum* (100% nucleotide sequence identity to Genbank accession number HG969192). The *gltA* gene nucleotide sequences of three *R. norvegicus* matched an uncultured *Bartonella sp.* clone ARC131 Genbank accession number JQ352095.1 (100% identity), previously detected in an *R. tanezumi* individual collected from Tholakeke, Gauteng Province, South Africa (Mostert 2008). *Bartonella* sequences from three individuals of *R. norvegicus* showed > 99 % sequence identity to an uncultured *Bartonella sp.* clone EZ8.6 Genbank accession number HM749297.1 previously identified in *M. namaquensis* sampled from Ezemvelo Nature Reserve, Gauteng Province, South Africa (Brettschneider *et al.* 2012). The final complete dataset consisted of 52 taxa including reference sequences from NCBI database and CIP isolates generated in-house (A.D.S. Bastos unpublished), was 435 nucleotides in length, and had 161 variable and 112 parsimony informative sites. The transition: transversion ratio (R) was 1.72 and the average nucleotide composition was T = 32.2%, C = 21.2%, A = 26.6% and G = 20.0%.

The ML, NJ and BI trees (Figure 1) recovered three lineages corresponding to two *Bartonella* species. Lineages II and III contained two *Bartonella* strains clustering with *B. elizabethae* (CIP103761), while lineage I corresponded to *B. tribocorum* of the Pasteur Institute's bacterial species ID number (CIP105476). Both species are considered zoonotic with the latter recently being implicated as the cause of febrile cases in patients in Thailand (Kosoy 2010). The phylogenetic trees (Fig. 2) showed that a group of fleas, ALX R26 (female), ALX R27 (female), ALX R128 (female), TEM R45 (male), TEM R53 (male) and TEM 53 (female) share the same *Bartonella* species as that of their host. Some fleas however, had a *Bartonella* species distinct from that of their hosts. This is shown by the clustering of ALX R19 (male), ALX R152 (male), TEM R43 (male) and TEM R49 (female) in lineages distinct from that of their hosts.

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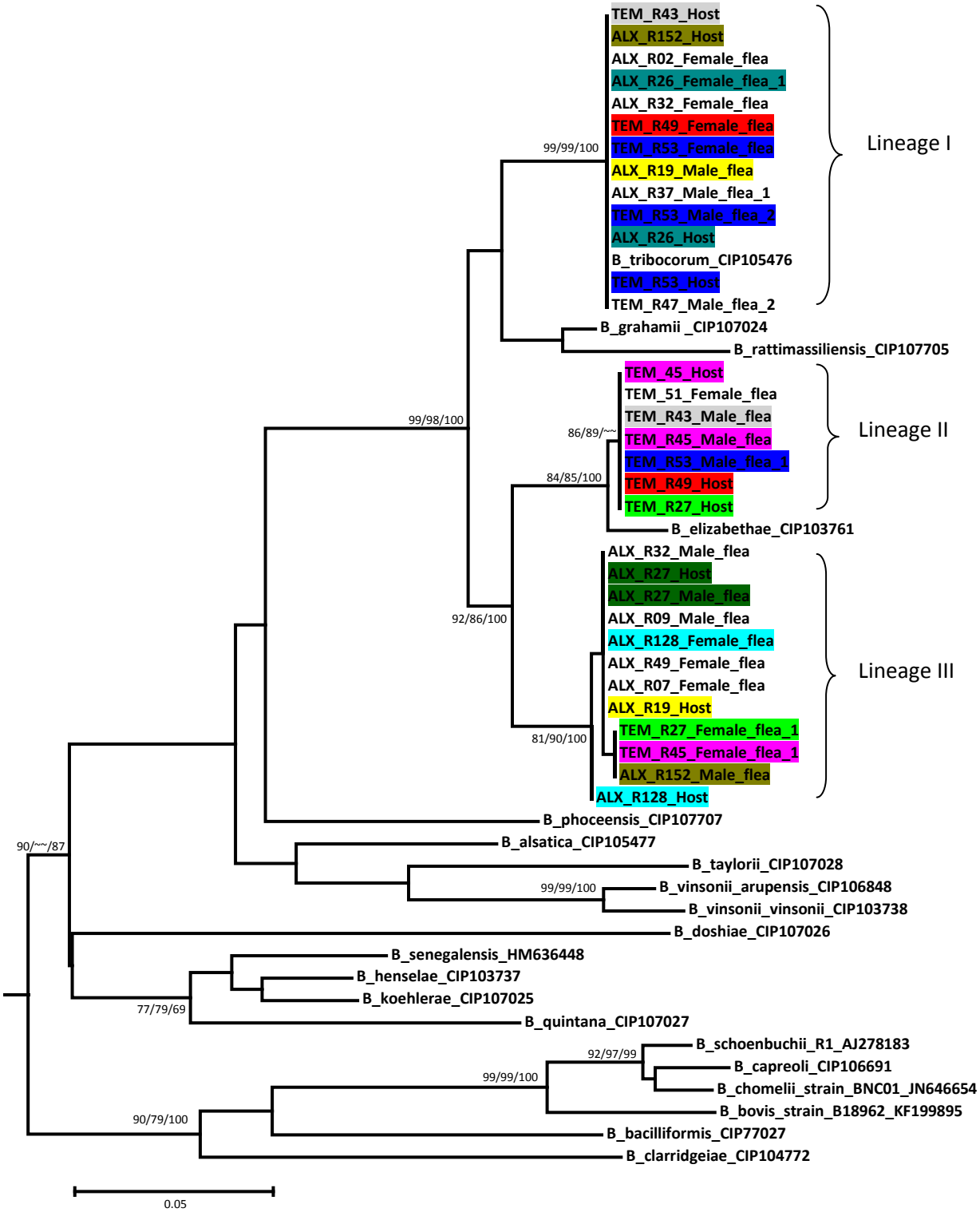

Figure 2: Maximum likelihood (ML) tree inferred using a partial citrate synthase gene segment (*gltA*) from *Xenopsylla brasiliensis* fleas and their hosts (*Rattus norvegicus)* sampled from the Alexandra and Tembisa townships in Gauteng Province, South Africa. Reference species names are followed by the corresponding Genbank accession number or CIP reference number. Nodal support values for (ML), Neighbour Joining (NJ) trees are based on 10,000 non-parametric bootstrap replications and are shown together with posterior probabilities from Bayesian analysis (BI) as ML/NJ/BI on the respective nodes.

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TEM R27 female flea harbours the same *Bartonella* species as its host, although the nucleotide sequences suggest that these were different strains. The tree also showed that fleas from the same host can harbour different *Bartonella* species. For example, TEM R53 was infested with three fleas of which two had a *Bartonella* species identical to that of their host (*B. tribocorum*) and one flea had a different *Bartonella* species (*B. elizabethae*). The same pattern is also shown for the TEM 45 host species from which one infected flea was found to contain the same *Bartonella* strain whereas the other flea was shown to be infected with a different *Bartonella* strain.

3.4 Discussion

Many studies have shown that arthropod vectors play a key role in *Bartonella* transmission for a broad range of mammalian hosts (Abbot *et al.* 2007; Bown *et al.* 2004; Cotte *et al.* 2008; Chomel *et al.* 2009; Tsai *et al.* 2011; Mediannikov *et al.* 2014). This study is one of the few studies in South Africa to investigate *Bartonella* in two rodent flea species *X. brasiliensis* and *X. cheopis* collected from infested *R. norvegicus* and *R. tanezumi* living in close proximity with humans. Amatre *et al.* (2009) studied flea diversity and infestation prevalence on rodents in Uganda and found that *R. rattus* collected in human dwellings were infested with *Xenopsylla cheopis* and *X. brasiliensis* in common with the findings in the present study.

These two flea species (*X. cheopis* and *X. Brasiliensis*) have been implicated as primary vectors of *Yersinia pestis* and *Murine typhus* (*Rickettsia typhi*) to humans in domestic and peri-domestic settings (Pollitzer 1954; Gratz 1999; Gage and Kosoy 2005; Bitman *et al.* 2010). Recently, Brettschneider *et al.* (2012) reported fleas to be the likely primary vectors of *Bartonella* in *Rattus* after developing a mathematical epidemiological model to investigate if differential ectoparasite loads in *R. rattus* and *R. norvegicus* could explain the observed differences in *Bartonella* prevalence for different *Rattus* hosts (Mostert 2010; Brettschneider 2011).

Xenopsylla brasiliensis and *X. cheopis* fleas collected in this study showed high levels of *Bartonella* prevalence (84.3%) supporting the importance of flea as the likely primary vector of *Bartonella* for *Rattus* from South Africa. However, no

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evidence of *Bartonella* infection was found in ticks. This could be because most of the ticks sampled in this study were larvae, and may have been sampled before getting a blood meal.

Bartonella elizabethae and *B. tribocorum* identified in *X. brasiliensis* in this study is a reason for concern since these two *Bartonella* species are known for their zoonotic potential. *Bartonella elizabethae* causes endocarditis while *B. tribocorum* causes febrile illness, with the latter's zoonotic potential being a recent discovery (Daly *et al.* 1993; Kosoy *et al.* 2010). A study in Los Angeles (U.S.A.) found identical *Bartonella* infection of *B. tribocorum* in *R. norvegicus* and their associated fleas, *X. cheopis* (Billeter *et al.* 2011).

Host switching has been reported for cosmopolitan fleas infesting small mammals (Amatre *et al.* 2009) and promotes the spread of *Bartonella* to a broader range of hosts whilst at the same time ensuring maintainenance of the infection in both host and arthropod vector populations. The present study shows that: i) fleas can harbour *Bartonella* species different to that carried by its host; ii) *Bartonella* can be detected in fleas collected from negative hosts, similar to findings in a study conducted by Gabriel *et al.* (2009); iii) fleas collected from the same host can harbour different *Bartonella* species, hence flea-pooling in molecular analysis should be discouraged; and iv) *Bartonella* in fleas can in some cases correspond to the *Bartonella* species present in the vertebrate host. These findings confirm previous conclusions that detection of *Bartonella* in fleas does not necessarily indicate infection in the host (Brettschneider 2011)

Many fleas have been reported to parasitize a narrow host range and are believed to be host-specific (Castle *et al.* 2004; Bai *et al.* 2008; Brinkerhoff 2008). However, in localities where *Bartonella* and host diversity is high and where fleas are taking blood meals from multiple hosts infected with different *Bartonella* species, mismatches between host and ectoparasite strains are more likely. This was confirmed in the present study which demonstrated that the same *Bartonella* species, *B. tribocorum*, *B. elizabethae* lineage II strain and *B. elizabethae* lineage III strains were shared amongst fleas and host species although fleas may not carry exactly the same *Bartonella* strain as the host. Several rodent species have been

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suggested to share some flea species (Buffet *et al.* 2013). In the present study, the overall *Bartonella* prevalence in *R. novegicus* was 57% slightly lower than that of the fleas. Blast results show that *Bartonella* strains characterised in this study are similar to *Bartonella* strains identified in a previous study in which *R. norvegicus* and *R. tanezumi* were sampled in different localities (Tholakele and O.R. Tambo International Airport, Gauteng Province, South Africa; Brettschneider 2011).

Co-infection and mixed *Bartonella* infections of 90% have been reported in flea vectors *Polygenis gwni* parasitizing cotton rats (*Sigmodon hispidus*) (Abbot *et al.* 2007), which was not the case with *X. brasiliensis* from the present study. Similar to previous mammalian studies, three arthropod haematophagous vectors (ticks, lice, fleas) (Chomel *et al.* 1996; Finkelstein *et al.* 2002; Fournier *et al.* 2002; Angelakis *et al.* 2010b; Mediannikov *et al.* 2014) were collected from *Rattus* species sampled in urban and peri-urban areas of Gauteng Province, South Africa. Lice were the most abundant ectoparasites and their *Bartonella* status still needs to be fully investigated. On the other hand, the potential of mites to carry *Bartonella* has been under-studied. Brettschneider (2011) found no evidence of *Bartonella* infection in a subset of mite samples collected from indigenous rodents in South Africa; however, this does not mean the same trend will hold for mites collected from invasive *Rattus* species. Further investigation should therefore be conducted since mites were the second-most abundant arthropods collected in this study.

Bartonella prevalence investigated in fleas, ticks and their hosts from this study has provided empirical estimates of *Rattus* host species and ectoparasite prevalence in South Africa. These data may be useful for incorporating in mathematical epidemiological models such as the one developed by Brettschneider *et al.* (2012) to study the dynamics of *Bartonella* transmission in *Rattus*. The findings in the present study are of public health concern since two species of zoonotic potential, *B.* elizabethae and *B. tribocorum* were identified.

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Chapter 4

4.1 General discussion

An exponential population increase in *Rattus norvegicus* numbers has been observed in urban environments, most notably in low-income neighbourhoods (Glass *et al.* 1988; Childs *et al.* 1998; Easterbrook *et al*. 2005; Glass *et al.* 2009; Himsworth *et al.* 2013; Kajdacsi *et al.* 2013; Costa *et al*. 2014; Himsworth *et al.* 2014). Abundant food resources, good harbourage systems and the non-removal of waste in these areas encourage the establishment of rats and as a result increased human-rat contact (Himsworth *et al.* 2013; Kajdacsi *et al* 2013). As known reservoirs for a wide range of pathogens including *Bartonella* (Easterbrook *et al.* 2007; Meerburg *et al.* 2009; Bastos *et al.* 2011; Himsworth *et al.* 2013) their presence has become a health risk to humans, their companion animals and livestock. Hence this study's aim was to obtain empirical estimates for key parameters that influence *Bartonella* transmission in three *Rattus* species (*R. rattus, R. tanezumi and R. norvegicus*) from peri-urban and urban areas in Gauteng Province, South Africa, with specific focus on determining rates of vertical transmission and evaluating the role of ectoparasites (Chapters 2 and 3).

The first part of the study which investigated the rate of vertical transmission of *Bartonella* in pregnant female *R. norvegicus* and their foetuses, focussed on informal settlements in Gauteng Province, South Africa. *Rattus norvegicus* was well-represented because of its abundance in informal settings and because it is one of the most successful invasive commensal rodents in the world. Similar to what has been observed in other countries, in South Africa *R. norvegicus* has been recorded to have the highest *Bartonella* infection prevalence compared to *R. tanezumi* and *R. rattus* (Ellis *et al.* 1999; Reeves *et al.* 2006; Hsieh *et al*. 2009; Mostert 2010; Brettschneider 2011; Tsai *et al.* 2010, Brettschneider *et al.* 2012).

Mostert (2010) recovered a *Bartonella* prevalence of 39% in *R. norvegicus*, 6.8% in *R. rattus*, and 4.7% in *R. tanezumi*. The same pattern was shown in a subsequent study conducted by Brettschneider (2011) in which rates of *Bartonella*

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infection were 27.2% for *R. norvegicus*, 5.55% for *R. rattus* and 7.34% for *R. tanezumi*. When modelling *Bartonella* infection in *R. norvegicus* and members of the *R. rattus* complex, a maximum difference of 4.6% is estimated from the SEI mathematical epidemiological model developed for this gram-negative bacterium, whereas the observed mean difference was 19% (Brettschneider *et al.* 2012). The difference in infection prevalence is suggested to be due to *R. norvegicus*' behaviour and body size since it has a larger body size, is aggressive and spends most of its time on the ground foraging, and digs burrows in the soil and is therefore more highly exposed to ectoparasites (Gratz 1997; Soliman *et al.* 2001; Traweger *et al.* 2006).

The present study recovered a high *Bartonella* prevalence (70.2%) in pregnant *Rattus* females. Only nine of the *Bartonella*-positive pregnant females had foetuses large enough to allow for torso dissections.The 87 foetuses screened for *Bartonell*a genome presence had an overall infection rate of 55.2%. Sequence comparisons revealed that similar *Bartonella* strains were present in the mothers and their foetuses. Results in this study confirm that vertical transmission of *Bartonella* occurs in naturally infected *R. norvegicus* in Gauteng Province, South Africa. Thus we reject the null hypothesis stating that there is no vertical transmission of *Bartonella* between invasive *Rattus* females to their offspring in urban and peri-urban areas in Gauteng Province, South Africa (Chapter 1). This is supported by previous observations by Kosoy *et al.* (1998) suggesting that transplacental transmission of *Bartonella* occurs in naturally infected cotton rats (*Sigmodon hispidus*) (58%) and in white-footed mice (*Peromyscus leucopus*) (36.84%).

This study is the first study to report on *Bartonella* detection in foetuses of invasive *Rattus* species and demonstrates that vertical transmission is one of the mechanisms used to maintain *Bartonella* infection in South African invasive *Rattus* species populations. This suggests that ticks, lice, mites, fleas and other known *Bartonella* vectors are not the only sources of infection in South African *Rattus* species populations and provides an additional biological parameter that may provide useful insights into the dynamics of *Bartonella* infection.

Two of the *Bartonella* species characterised in this study are zoonotic pathogens associated with rodents. Their *Bartonella* sequences cluster with *B.*

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elizabethae of the Pasteur Institute's bacterial species ID number (CIP103761) and *B. tribocorum* of the Pasteur Institute's bacterial species ID number (CIP105476) generated in-house (Bastos unpublished) which are known to respectively cause endocarditis and febrile illness in humans (Buffet *et al*. 2013; Figure 3; Chapter 2). This is of primary concern considering that people residing in the sampled areas generally have higher levels of HIV-positivity and that people whose immune system are compromised are more likely to get opportunistic *Bartonella*-related infections (Koehler *et al.* 2003).

The second part of the study attempted to identify arthropod ectoparasites infesting *Rattus* species in urban and peri-urban areas of Gauteng Province, South Africa in order to determine their vector potential. In general, *Bartonella* infection dynamics and transmission routes in *Rattus* species are understudied in South Africa. *Bartonella* transmission is, however, generally known to occur through biting, scratching, and contact with urine and faeces of infected haematophagous arthropod vectors such as ticks, fleas, mites and sandflies (Bai *et al.* 2009; Chomel *et al.* 2009), and as demonstrated in the present study by vertical transmission.

In order to address the vector-borne aspects of *Bartonella* transmission in *Rattus*, we collected a total of 1400 ectoparasites from 450 *Rattus* specimens. Ectoparasites sampled in this study included fleas, mites, lice and ticks. Identified ectoparasites included *Xenopsylla brasiliensis* and *X. cheopis* fleas (*n* = 112), *Haemaphysalis elliptica* ticks (*n* = 25), *Ornithonyssuss bacoti* and *Laelaps spp.* mites (*n* = 392). Although all ticks sampled from *R. norvegicus* and *R. tanezumi* were screened for *Bartonella* genome presence no evidence of infection was found. Fleas were only sampled from live-caught *R. norvegicus.* Of the 112 fleas sampled, 108 were screened for *Bartonella* genome presence and an overall prevalence of 84.3% was identified. Thus incombination with the 0% prevalence, an overall tick-flea *Bartonella* prevalence of 68.4% was identified which is higher than the 54.1% prevalence determined for *Rattus* hosts co-infected with both ectoparasites. Although this study focussed on the two ectoparasite types (ticks and fleas) considered in the original model (Brettschneider et al. 2012), the abundance of other ectoparasite types prompted a pilot study on *Bartonella* genome presence in mites. A *Bartonella* prevalence of 16.6% was identified in a subset of 12 mites, suggesting

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that further studies are warranted as this was the second-most abundant ectoparasite group recovered from urban rats.

Phylogenetic analysis of 24.2% ($^{22}/_{91}$) of the PCR-positive fleas and 25% ($^{10}/_{40}$) of their PCR-positive *Rattus* hosts revealed three distinct lineages corresponding to two *Bartonella* species Lineages II, III, and *B. elizabethae* CPI 103761 clustered together with a 92% and 98% bootstrap support (ML and NJ, respectively), while lineage I and *B. tribocorum*_CIP105476 clustered together with 99% bootstrap support for both ML and NJ analysis (Figure 2; Chapter 3). A BI of 100% was shown for all three lineages (Figure 2; Chapter 3). NCBI blast search results of lineages II and III sequences clustering with *B. elizabethae* CIP 103761 showed between 99% and 100% blast similarities to *Bartonella* previously identified in the Namaqua rock mouse (*Micaelamys namaquensis*), and in R*. tanezumi* and *R. norvegicus* sampled in Gauteng Province, South Africa. As *Micaelamys namaquensis* is an endemic rodent species capable of attaining pest status (Muteka *et al*. 2006) and is widely distributed throughout the South African subregion (Skinner and Chimimba 2005) it is probably that in a commensal setting that ectoparasites are shared between this native species and invasive rats. The detection of *Rattus*-associated zoonotic *Bartonella* strains in native species, including *M. namaquensis,* sampled from natural (Pretorius *et al.* 2004) and commensal settings (Bastos 2007) suggest that flea-assisted exchanges of *Bartonella* strains between invasive and endemic species.

Results from the ML, NJ and BI trees indicated that approximately 50% of the fleas have the same *Bartonella* species as their host and the other 50% are infected with a *Bartonella* species that differs from that of their host. The latter concurs with previous reports of flea-host rates of compatibility (Foil *et al*. 1998; Finkelstein *et al.* 2002; Engel and Dehio 2009) and also suggests that fleas feed on numerous hosts, increasing the chance of multiple infections of *Bartonella* (Brinkerhoff *et al.* 2010; Buffet *et al.* 2013). *Bartonella* prevalence in fleas was 84.3% whereas that of their host (*R. norvegicus*) was 59.4%. None of the ticks were found to harbour *Bartonella.* The overall prevalence for *Bartonella* in the ectoparasite subset, comprising of ticks and fleas, was 68.4%, ($^{91}/_{133}$) and with the 12 mites incorporated, it was 64.1% ($^{93}/_{145}$). We therefore reject the null hypothesis stating that the prevalence rates of

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Bartonella infection in *Rattus*-associated ectoparasites is the same as the prevalence of *Bartonella* infection in *Rattus spp.*

This study provides *Rattus*-specific data that can be used in understanding the transmission dynamics of *Bartonella* infections in urban and peri-urban settings in South Africa. The results in the present study will contribute towards an increasing body of knowledge on vector-borne diseases in Africa, and may assist with mitigating *Bartonella* infections. Understanding *Bartonella* transmission dynamics is crucial, more especially since *Bartonella* species previously isolated from invasive commensal rodents are known for their zoonotic potential (Daly *et al.* 1993; Kosoy *et al.* 2010).

Brettschneider *et al.* (2012) developed a mathematical epidemiological model (see Figure 1 in Chapter 1) involving two hosts (*R. rattus* and *R*. *norvegicus*) and two vectors (*Ixodid* ticks and *Xenopsylid* fleas) in order to study the dynamics of *Bartonella* infections in South Africa. The aim of the model was to study mathematically the relationship between the contributing factors of *Bartonella* infection in a commensal rat population in South Africa in an effort to explain differences in the infection rates between two different species of *Rattus* with respect to their ectoparasites infestation (Brettschneider *et al.* 2012). However, the model lacked information on key biological parameters emphasizing the need for more detailed and extensive research on *Bartonella* infections in South African *Rattus* species. Consequently,the *Rattus*-specific estimates determined in the present study can be used to refine the epidemiological mathematical model developed by Brettschneider *et al.* (2012) by replacing biological estimates based on data from peer-reviewed studies on unrelated rodent hosts that was used in the original model (Gratz 1997; Kosoy *et al.* 1999; Soliman *et al.* 2001; Kim *et al.* 2005).

Rattus norvegicus is more widespread and has much higher rates of *Bartonella* infection than *R. tanezumi* and *R. rattus* in South Africa (Mostert 2010; Brettschneider 2011). Hence, *R. norvegicus*-specific data generated in this study will contribute to refining the model developed by Brettschneider *et al.* (2012). This is important since the epidemiological mathematical modelling of gram-negative

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bacteria in the two *Rattus spp.* and their ectoparasites was the driver for the present study.

The potential for horizontal and the occurrence of vertical transmission of two *Bartonella* species *(B. elizabethae and B. tribocorum)* was confirmed in the present study. The transmission efficiencies were, however, different. The results from this study indicate for the first time that *Bartonella tribocorum* appears to be transmitted more efficiently through the vertical transmission route whereas *B. elizabethae* appears to be transmitted more efficiently through flea-vector transmission. Vertical transmission studies in naturally infected *Rattus* species are challenging, as they require sampling of pregnant *Rattus* females with sufficiently well-developed fetuses to permit torso dissections. Additional *Rattus*-specific data are needed in order to broaden our understanding of this aspect of *Bartonella* transmission in invasive *Rattus* species from South Africa.

Four *Rattus*-associated ectoparasites groups were sampled in this study; similar to what was found in the Namaqua rock mouse (*Micaelamys namaquensis* from South Africa (Fagir *et al.* 2014). These included fleas, ticks, mites and lice; however, for logistical reasons, only fleas, ticks and mites were identified. The focus on fleas and ticks was guided by the results of an epidemiological model developed by Brettschneider *et al.* (2012) which investigated whether differential ectoparasite load of these two ectoparasite types fully explains the observed differences in *Bartonella* prevalence in *R. rattus* and *R. norvegicus.*

Lice were the most abundant *Rattus*-associated ectoparasites sampled (*n* = 871), and as competent vectors for *B. quintana*, the causative agent for trench fever (Bruce *et al.* 1921; Mooser and Weyer 1953; Vinson *et al.* 1969; Raoult and Roux 1999; Fournier *et al.* 2001; Bonilla *et al.* 2014), further studies should be conducted in which lice are identified to species level and assessed for *Bartonella* presence and vector competence. The second-most abundant *Rattus*-associated ectoparasites were mites, *Ornithonyssuss bacoti* and *Laelaps spp.* (*n* = 392). A pilot study in which 12 randomly selected individuals (n=9 *Laelaps* and n=3 *Ornithonyssuss*) were evaluated for *Bartonella* genome presence revealed that two of the nine *Laelaps* mites were PCR-positive and that further investigations are warranted.

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In conclusion, this study demonstrates that *B. elizabethae* and *B.tribocorum* are transmitted vertically and horizontally (*via* blood sucking arthropod vectors - primarily fleas) in invasive *R. norvegicus* in South Africa. It further appears that the efficiency which different *Bartonella* species are transmitted may be influenced by the mode of transmission mode. The results further confirmed that fleas play a major role in *Bartonella* transmission.

4.2 Recommendations

1. For future studies, experiments involving breeding of *Rattus* species and monitoring of foetus development should be done before dissections of female pregnant *Rattus* in order to facilitate vertical transmission studies. Furthermore, the possibility of *Bartonella* DNA fragments passing through placental tissue should be considered.
2. More comprehensive research on *Bartonella* infection in *Rattus*-associated ectoparasites needs to be done in South Africa, incorporating all ectoparasites collected.

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4.4 APPENDIX I

Study permits

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

ANIMAL USE AND CARE COMMITTEE
Private Bag X04
0110 Onderstepoort

Tel +27 12 529 8434 / Fax +27 12 529 8300
e-mail: aucc@up.ac.za

Ref: EC025-10

30 June 2010

Prof CT Chimimba
Department of Zoology & Entomology
University of Pretora
(ctchimimba@zoology.up.ac.za)

Dear Prof Chimimba

EC025-10: The dynamics between rodent-borne diseases and their reservoir hosts: The case of the invasive *Rattus* from southern Africa *(R Jiulius)*

The application for ethical approval, dated 14 June 2010 was approved by the Animal Use and Care Committee at its meeting held on 28 June 2010.

Best regards

E Mostert

Elmarie Mostert

AUCC Coordinator

Copy: Ms A O'Niel
R Julius

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agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Private Bag X138, Pretoria, 0001
Delpen Building, c/o Annie Botha & Union Street, Riviera, 0084

From: Directorate Animal Health
Tel: 012 319 7502
Fax: 012 319 7470
E-mail: ThaboMo@daff.gov.za
Enquiries: Mr. Thabo Motsisi
Our Ref: 12/11/1/1/8
Your Ref No : 13788 – Rolanda Julius

Date: 21 June 2010

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

Dear Prof. C.T.Chimimba

Your fax / memo / letter dated 02 June 2010, requesting permission under Section 20 of the Animal Diseases 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:

Title of research/study: The dynamics between rodent-borne zoonotic diseases and their reservoir hosts: the case of the invasive Rattus from Southern Africa.
Researcher (s): Rolanda Julius
Institution: University of Pretoria, Department of Zoology
Your Ref. Number: 13788 – Rolanda Julius Project Protocol
Our ref Number: 12/11/1/1/8

Kind regards

DF KEET

ACTING DIRECTOR: ANIMAL HEALTH

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PREMIER OF THE PROVINCE OF GAUTENG
NATURE CONSERVATION

CPF6 No. 0032

PERMIT TO HUNT AND/OR COLLECT AND CONVEY A WILD ANIMALS FOR SCIENTIFIC PURPOSES

Issued in terms of the provisions of the Nature Conservation Ordinance, 1983 (Ordinance 12 of 1983)

Name of permit holder: Miss Anja Le Grange

Residential address: The Fields BC 415
Burnette Street 1066
Hatfield

DATE STAMP
GAUTENG PROVINCIAL GOVERNMENT
DEPARTMENT OF AGRICULTURE
CONSERVATION, ENVIRONMENT
2010 -05- 11
DIRECTORATE OF CONSERVATION
PERMITS OFFICE
PO BOX 8769, JOHANNESBURG 2000

Name and address of institution or department on whose behalf shall be hunted and conveyed:
DEPARTMENT OF ZOOLOGY & ENTOMOLOGY,
UNIVERSITY OF PRETORIA, PRETORIA, 2000

PARTICULARS OF WILD ANIMALS WHICH MAY BE HUNTED AND CONVEYED

Number	Species	Sex
The Permit to Capture, release, convey and keep		
Specimens of Small mammals.		
Species are as per attached addendum.		
Conditions are attached.		

In terms of and subject of the provisions of the **Nature Conservation Ordinance, 1983 (Ordinance 12 of 1983)** and the regulations framed thereunder, the abovementioned person is hereby authorised, subject to the conditions appearing on this permit to hunt and convey the wild animal/s referred to above during the period of validity of this permit on behalf of the institution or department referred to above.

PARTICULARS IN CONNECTION WITH THE HUNT

Number	Species	Sex	Date hunted and conveyed

SIGNED ON BEHALF OF THE PREMIER

SIGNATURE OF PERMIT HOLDER

This permit is valid from 11/05/2010 - 10/05/2011

(See conditions on reverse side)

the-blackhand 011 435 2800 (0064 - CPF6)

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Addendum to CPF6- 0032

Miss Anja Le Grange
The Fields BC 415
Burnette Street 1066
Hatfield
0083

Common Name	***Scientific Name***
Multimammate mouse	***Mastomys natalensis***
Sripsed mouse	***Rhabdomys pumilio***
Namaqua rock mouse tete veld rat	***Aethomys namaquansis***
Tete veld rat	***Aethomys ineptus***
Spiny mouse	***Acomys spinosissimus***
Bushveld gerbil	***Tatera leucogaster***
Highveld gerbil	***Tatera brantsii***
Grey climbing mouse	***Dendromys melanotis***
Angoni vlei rat	***Otomys angoniesnsis***
Pygmy mouse	***Mus indutus***
Asian house rat	***Rattus tanezumi***
Black rat	***Rattus rattus***
Brown rat	***Rattus norvegicus***

FOR PREMIER _____

GAUTENG PROVINCIAL GOVERNMENT
DEPARTMENT OF AGRICULTURE
CONSERVATION, ENVIRONMENT
2010 -05- 11
DIRECTORATE OF CONSERVATION
PERMITS OFFICE
PO BOX 8769, JOHANNESBURG 2000

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Conditions to CPF6- 0032

Miss Anja Le Grange
The Fileds BC 415
Burnette Street 1066
Hartfield

- This permit is valid for one year and permits the capture of only rodents only within the Gauteng Province.
- Permission must be obtained from the land owner for any captures to be conducted in private land.
- Animals must be kept in a laboratry environment that does not differ Extensively form their natural environment.
- All surgical procedures to be conducted on the speciemens must be done by a Sutaibly qualified veterinarian. Animals must be euthanized in a humane way.
- The applicant must provide tha Gauteng Nature Conservation Directorate with The lists of all small mammal species and geo-referenced localities of any Threatened species encounted under this permit on an annual basis; failing Which the applicant for the renewal of the permit might not be used successful.
- A copy of any publications resulting from this research must be submitted to The mammal scientists at the Department of Agriculture & Rural Development.

FOR PREMIER: Mchunu

GAUTENG PROVINCIAL GOVERNMENT
DEPARTMENT OF AGRICULTURE
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2010 -05- 11
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