Bartonellae of the Namaqua rock mouse*, Micaelamys namaquensis* (Rodentia: Muridae*)* from South Africa

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ABSTRACT

The aim of this study was to determine *Bartonella* prevalence and diversity in Namaqua rock mice, *Micaelamys namaquensis*, a species endemic to South Africa which can attain pest status. A total of 100 heart samples collected monthly from March to December were screened for *Bartonella* genome presence using three primer sets targeting the citrate synthase (*gltA*) gene, the NADH dehydrogenase gamma subunit (*nuoG*) gene and the RNA polymerase β-subunit-encoding gene (*rpoB*). An overall prevalence of 44% was obtained, with no statistically significant differences or correlations between infection rates and rodent sex, month of capture or season of capture. Phylogenetic analysis of 34 unambiguous *gltA* sequences revealed the presence of three discrete *Bartonella* lineages in *M. namaquensis*, one of which corresponds to *B. elizabethae*, a species with known zoonotic potential.

Keywords: *Bartonella*, *Micaelamys namaquensis*, prevalence, diversity, Namaqua rock mouse, South Africa.

Introduction

Studies on the gram-negative bacterial genus *Bartonella* have shown prevalence in rodent species can be as high as 70%, with an average of around 50% in natural populations (Kosoy *et al.* 2004 a and b). More specifically, murid rodents have been shown to carry a wide spectrum of *Bartonella* species including *B. elizabethae, B. grahamii, B. tribocorum, B. rattimassiliensis, B. rattiaustraliani, B. taylorii, B. doshiae, B. tribocorum B. phoceensis, B. cooperplainensis, B. silvatica, B. japonica* and *B. queenslandensis* (Kosoy *et al*. 2004; Gundi *et al.* 2009; Inoue *et al.* 2010). Studies conducted on rodents in the northern hemisphere suggest that transmission mostly occurs either during summer and autumn months or during middle and late reproductive periods (Fichet-Calvet *et al.* 2000; Morway *et al.* 2008).

Two studies documenting the prevalence and diversity of *Bartonella* infections in murid rodents from South Africa, have laid the foundations for investigations on ecological and epidemiological aspects of *Bartonella* infections. The first study assessed *Bartonella* prevalence and infection rates in natural populations of eight endemic rodent species that included the Namaqua rock mouse (*Micaelamys namaquensis*) (Pretorius *et al*. 2004). The second study (Bastos 2007) focused on *Bartonella* prevalence in eight endemic, but commensal rodent species, also inclusive of *M. namaquensis*. These studies reported infection rates with *Bartonella* of 44% (Pretorius *et al.* 2004) and 56% (Bastos 2007) and shared bartonellae lineages between rodent species such as *M. namaquensis*, the bushveld gerbil *(Gerbilliscus leucogaster*) and the four striped grass mouse (*Rhabodomys pumilio*).

The present study evaluates *Bartonella* infection dynamics in *M. namaquensis*, a southern hemisphere murid rodent species that periodically attains pest status, when occurring with humans (Skinner and Chimimba 2005), and represents the first investigation into the dynamics of *Bartonella* in a single murid rodent species from South Africa.

Materials and Methods

Sampling and study area

The present study was based on 100 individuals (57 females, 40 males and 3 not sexed) of *M. namaquensis* sampled monthly over a 10-month period (10 individuals per month) between March and December 2002 at Ezemvelo Nature Reserve (South Africa). Organ samples were dissected using sterile equipment and stored at -20 °C until 2008, when the genomic DNA extractions were performed. The reproductive period of the Namaqua rock mouse, which is widely distributed throughout the southern African subregion, falls primarily within the rainy season spanning the months October to March and coinciding with the Spring and Autumn months (Muteka *et al.* 2006). Animal trapping, euthanazation, dissection and ethical guidelines are detailed in the reproductive physiology study of Muteka *et al.* (2006) which provided the impetus for sample collection.

Molecular detection, characterisation and phylogenetic analysis

Genomic DNA was extracted from 50mg of heart tissue using the Roche High Pure DNA extraction kit (Roche Diagnostics, U.K.) according to manufacturer specifications. All samples were screened for *Bartonella* genome presence using three primer sets, viz. (i) Bart-EF (CACGACTCYATTGATATTACAGA) and Bart-ER (GCACGTGGRTCATAATTTTTATA) which target a 513 bp region of the citrate synthase gene (*gltA*) (Bastos 2007, Bastos in prep.), (ii) the NADH dehydrogenase gamma subunit (*nuoG*) primers (Colborn *et al.* 2010) which produce

a 346 bp amplicon, and (iii) the RNA polymerase β-subunit (*rpoB*) primers that amplify a 410 bp region of the target gene (Drancourt and Raoult 1999). Cross-contamination was minimised by doing pre- and post-PCR in separate rooms and by preparing PCR reactions in a DNA-free hood, subsequent to UV decontamination. One negative reaction control was included for every five samples, as advocated by Fenollar and Raoult (2004) and all PCR reactions were run on the same thermal cycler (ABI 2720, Applied Biosystems). Each sample was screened at least twice with the *gltA* primer set, and assigned positive-*Bartonella* genome status on the basis of corresponding amplification of *nuoG* and *rpoB* gene targets.

All *gltA* gene positive samples were purified using the Roche PCR Product Purification Kit, and cycle sequenced using BigDye v. 3.1 terminator cycle-sequencing kit (Perkim-Elmer, Foster City, U.S.A. The *nuoG* and *rpoB* amplicons of two samples that were negative for *gltA* gene amplification, but positive for these gene regions were sequenced to confirm *Bartonella* genome presence. Samples were run on an ABI 3130 sequencer and the resulting sequence chromatograms were viewed and edited in the Chromas programme which is embedded in Mega 4 (Tamura *et al.* 2007) prior to performing a BLAST nucleotide search (www.ncbi.nm.nih.gov/blast) to identify the *Bartonella* species with the highest sequence identity. All *gltA* nucleotide sequences generated in this study, and those representative of 12 reference sequences were aligned using the ClustalX (Thompson *et al*. 1997, Chenna *et al.* 2003) function incorporated in Mega 4 and have been submitted to Genbank under accession numbers (HM749283, HM749286, HM749288, HM749291-93, HM749297-310).

Distance trees were inferred in Mega 4 (Tamura *et al.* 2007) and MrBayes v3.1 (Heulsenbeck and Ronquist 2001) was used for Bayesian inferences (BI). The best fit model of sequence evolution was selected under the Akaike Information Criterion (AIC) in jModeltest (Posada *et al.* 2008). Nodal support for the Minimum Evolution (ME) tree was assessed through 1000 non-parametric bootstrap replications. BI was run over one million generations, after which 10% of the trees were discarded as burn in.

Statistical Analyses

Statistical correlations between *Bartonella* prevalence data and (1) rodent sex (excluding 3 unsexed individuals), (2) month, (3) season of capture and (4) reproductive period were tested using General Linear Models. The Kolmogorov-Smirnov test was used to test differences in prevalence for wet *vs.* dry season and reproductive *vs.* non-reproductive season. The reproductive period of the Namaqua rock mouse falls primarily within the rainy season spanning the months October to March and coinciding with the Spring and Autumn months as defined by Muteka *et al.* (2006). Rainfall data were obtained from Swanepoel and Bredenkamp (2006). All statistical analyses were conducted in Statistica version 10 (StatSoft Inc. USA 2010).

Results

Genetic analyses

PCR amplification of the three gene regions as well as sequencing of the *gltA* region were used to assign unequivocal *Bartonella* status and identified bacterial genome presence in 44 samples. A high proportion ($^{8}/_{44}$; 18%) of ambiguous *gltA* sequences (based on multiple peaks observed in the sequence diagram) were recovered and although not included in the phylogenetic analyses, were included in all statistical analyses due to independent confirmation of *Bartonella* genome presence provided by corresponding amplification and selected sequencing of the *nuoG* and *rpoB* gene targets. Additionally, two samples (EZ7-4 and EZ12-8) initially assigned negative *Bartonella* status following *gltA* screening, were found to be positive by n*uoG* and r*poB* gene amplification and sequencing. These samples were included in the statistical analyses, but not the phylogenetic analyses.

Phylogenetic analyses were performed on a 46-taxon *gltA* gene dataset comprising 34 *Bartonella*-positive *M. namaquensis* sequences, and 12 *Bartonella* reference strains obtained

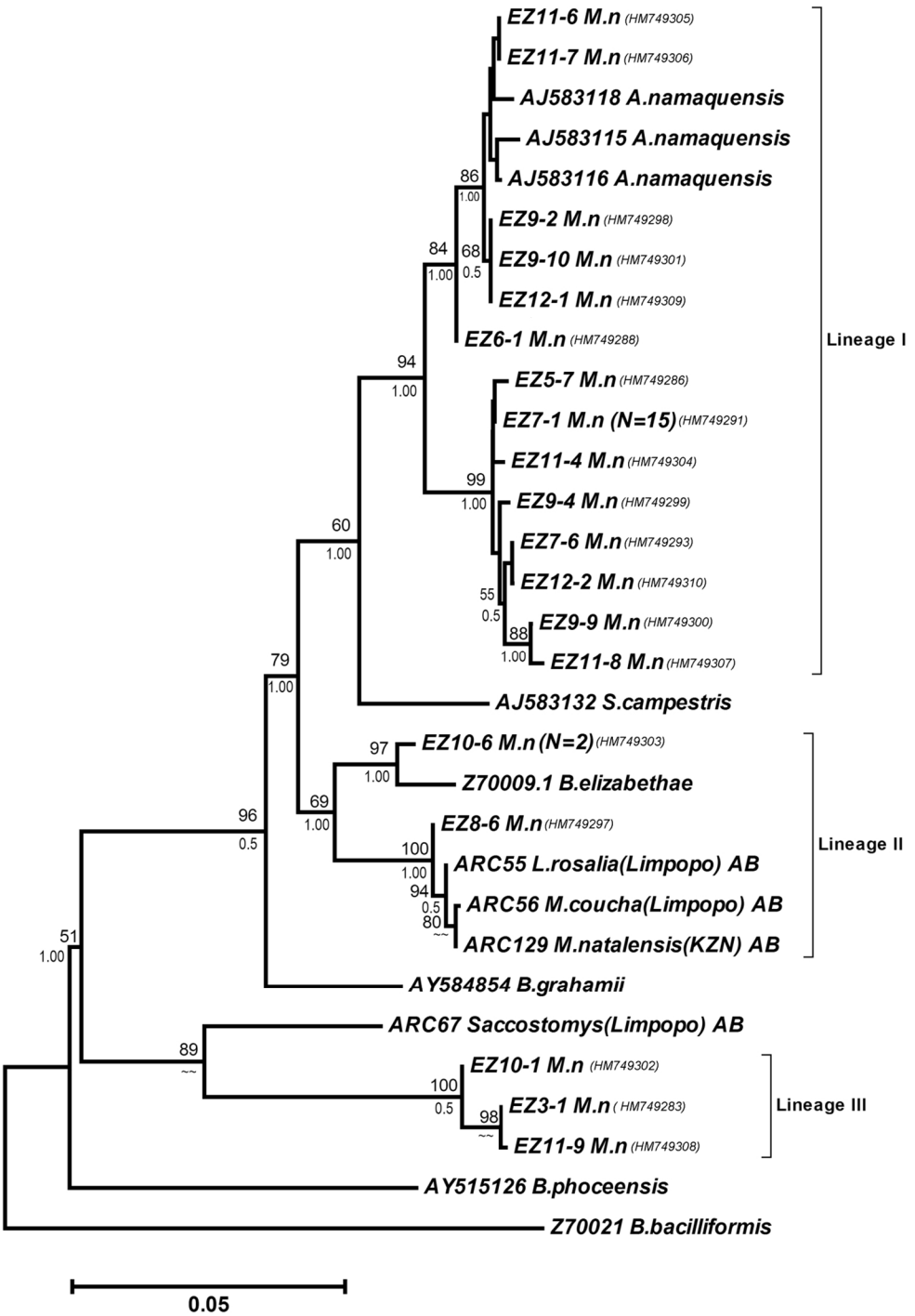
from the National Centre for Biotechnology Information (NCBI) database and from a previous study (Bastos 2007). Genbank accession numbers for all reference sequences are included in the taxon name (Figure 1). The TIM1+I+G model of sequence evolution with gamma parameter = 0.427 was chosen as the best fit model in jModeltest under the AIC. Results from ME and BI recovered the same tree topology and the bootstrap and support indices are summarised on the branches of the ME tree displayed in Figure 1.

Three distinct lineages, representing three distinct species of *Bartonella* based on a 0.05 - 0.11 mean uncorrected pairwise genetic distance between the lineages (see also Birtles and Raoult 1996) were recovered. Genetic distances between these lineages and known *Bartonella* species ranged from 0.02 to 0.12. Lineage I, consisting of 28 *Bartonella* amplicons (of which 15 are identical and represented by sample EZ 7-1_MN) from this study, corresponds to the *Bartonella* species identified in three *M. namaquensis* individuals from the study by Pretorius *et al.* (2004) (AJ583115, -116 and -118), suggestive of an *M. namaquensis*-specific clade. Lineage II consists of three *Bartonella* amplicons (of which two are identical and represented by sample EZ10-6_MN) that cluster with *B. elizabethae* (1-3 % similarity), a zoonotic bacterium often found in commensal rodents. This clade also includes *Bartonella* sequences identified in individuals from the genera of multimammate and striped mice of the *Mastomys* and *Lemniscomys* genera (Bastos 2007; Figure 1). The third lineage, comprising three *Bartonella* amplicons, represents a genetically-distinct and novel species which is sister to a bacterial lineage previously found in the pouched mouse *Saccostomus campestris* (Bastos 2007), however, Bayesian support for this grouping was low.

Bartonella prevalence in relation to host sex, season, rainfall, and reproductive period

An overall prevalence of 44% was recovered for the 100 *M. namaquensis* individuals screened by PCR. Males had higher average prevalence of 52.5% ($^{21}/_{40}$), with 38.5% ($^{22}/_{57}$) of females testing positive over the entire period. Monthly prevalence ranged from 20% to 70%

Figure 1. Minimum Evolution (ME) tree inferred from partial *gltA* gene sequences of *Bartonella* occurring in 34 *Micaelamys namaquensis* sampled monthly over a 10-month period at Ezemvelo Nature Reserve (Mpumalanga Province) in South Africa. Bootstrap and support indices are indicated for ME (above) and BI (below). All individuals from this study are prefixed with an EZ. Individuals with alternate codes correspond to samples from either the studies by Pretorius *et al.* (2004) (AJ583115-116, 118) and Bastos (2007) (ARC), or are Genbank-acquired sequences.



(Table 1). No difference was observed between wet and dry season prevalences, and although infection levels were slightly higher during the reproductive season, these differences were not statistically significant ($P > 0.1$) (Table 1). There was also no statistically significant difference between the proportion of infected rodents either between different months, between different seasons (Month: $\chi^2 = 2.44$; d.f. = 99; $n = 100$; $P = 0.37$; Season: $\chi^2 = 1.71$; d.f. = 99; $n = 100$; $P = 0.07$) or between rodent sex ($\chi^2 = 0.45$; d.f. = 96; $n = 97$; $P = 0.17$).

Discussion

From the phylogenetic analysis and comparison with bartonellae formerly reported in *M. namaquensis* and rodents in general, we observed a three lineage assemblage of bartonellae within the population assessed in the present study. There appears to be some specialization of these bacteria within this host as the largest proportion of bartonellae clustered within an *M. namaquensis*-dominated lineage. This lineage also includes three isolates from a previous *M. namaquensis* study conducted in the centrally-located Free State Province of South Africa (Pretorius *et al.* 2004), confirming that the host species carriage of this *Bartonella* species is applicable to other geographical areas in South Africa. Conversely, the phylogeny indicated the presence of *Bartonella* strains that lack host-specificity, occurring not only in *M. namaquensis* but also in other endemic rodent species as well as invasive rodents in the area. In particular, lineage II clustered with *bartonella*e previously isolated from invasive *Rattus* species, from endemic species belonging to the genera *Mastomys* and *Lemniscomys* (Bastos, 2007) and with a zoonotic reference sequence (Fig. 1). The broad host species range suggests that generalist ectoparasites, such as fleas, ticks and lice (see Zumpt *et al.* 1966) most likely feed on these species, facilitating spread of the bacterium between diverse rodent hosts. The recovery of *B. elizabethae* in *M. namaquensis* is of particular relevance as this bacterial species has been associated with infective endocarditis in humans (Daly *et al.* 1993), and *M. namaquensis* is a

Table 1. *Bartonella* infection in *Micaelamys namaquensis* sampled monthly over a 10-month period at Ezemvelo Nature Reserve (Mpumalanga Province) in South Africa. Percentage infections per month and season are shown with average rainfall data*.

Month/year	Rainfall	No. pos / total	Seasonal prev.	Prev. by rainfall	Prev. by reproductive season
March 2002	74mm	$^{3}/_{10}$	*Autumn 33.3%*	**Wet season 44%**	**Reproductive 45%**
April 2002	46mm	$^{4}/_{10}$			**Non-reproductive 43.3%**
May 2002	12mm	$^{3}/_{10}$		**Dry season 44%**	
June 2002	7mm	$^{6}/_{10}$	*Winter 46.6%*		
July 2002	2mm	$^{5}/_{10}$			
August 2002	5mm	$^{3}/_{10}$			
September 2002	17mm	$^{5}/_{10}$	*Spring 60%*		
October 2002	74mm	$^{7}/_{10}$		**Wet season 44%**	**Reproductive 45%**
November 2002	102mm	$^{6}/_{10}$			
December 2002	89mm	$^{2}/_{10}$	*Summer 20%*		

*Data obtained from Swanepoel & Bredenkamp (2006).

known agricultural pest that can reach very high population numbers in rural human settlements (Muteka *et al.* 2006). When rodent population irruptions occur, they involve unusually high reproductive rates (corresponding to food abundance such as crop harvests) and subsequent high mortality rates (associated with over-exploitation of food resources) (Muteka *et al*. 2006).

The high-proportion of mixed-sequences (18% of the *gltA Bartonella*-positive samples), detected in this study, is not uncommon, and concurs with the levels reported in culture and other PCR-based studies (Birtles *et al.* 2001; Pretorius *et al.* 2004; Holden *et al.* 2006; Abbot *et al.* 2007). Similarly, the average infection prevalence of 44% in *M. namaquensis* falls within the range reported for other wild rodents (see Kosoy *et al.* 2004 a and b; Jones *et al.* 2008). Although not statistically supported in this study, seasonal variation in the transmission of *Bartonella* in rodents has been reported previously in two northern hemisphere studies (Fichet-Calvet 2000; Morway *et al.* 2008).

The higher infection rate observed during the non-reproductive period of *M. namaquensis* could be related to rodent behaviour and ectoparasite abundance during different seasons of the year. Rodent host behaviour changes with the changing season, which may affect the likelihood of exposure to ectoparasites and their associated diseases (Gratz 1954; Soliman *et al.* 2001). Vector-borne diseases such as bartonellae are, therefore, anticipated to show some degree of variation throughout the year as the lifecycle of the vector and host changes with season (Chamberlin *et al*. 2002). The reproductive periods of the year as stipulated here, however, are not directly correlated with reproductive status of the individuals and differences between reproductive and non-reproductive seasons were not statistically significant. Rodent sex, month of capture, and monthly rainfall were also evaluated for their possible contribution to fluctuations in infections, but no statistically significant correlations were found. Differences observed between infection prevalences in males and females could be related to differences in male and female social behaviour as well as hormonal effects on immune system function (Klein 2000). Gender-bias has been reported for potential arthropod

vectors (ticks) of *Bartonella*, with more females infected than males (Halos *et al.* 2004; Holden *et al.* 2006), but no such difference- has been reported for a wild population of rodents.

In conclusion, this study revealed *M. namaquensis* to host at least three lineages of bartonellae, one of which has known zoonotic potential. Preliminary indications that environmental and demographic factors may influence *Bartonella* infection rates of *M. namaquensis* require further investigation through larger scale studies on this species.

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Author Disclosure Statement

No competing financial interests exist.

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