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Identification of Diverse *Bartonella* Genotypes among Small Mammals from Democratic Republic of Congo and Tanzania

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Abstract. Small mammals from the Democratic Republic (DR) of the Congo and Tanzania were tested to determine the prevalence and genetic diversity of *Bartonella* species. The presence of *Bartonella* DNA was assessed in spleen samples of the animals by *rpo*B- and *glt*A-polymerase chain reactions (PCRs). By *rpo*B-PCR, *Bartonella* was detected in 8 of 59 animals of DR Congo and in 16 of 39 Tanzanian animals. By *glt*A-PCR, *Bartonella* was detected in 5 and 15 animals of DR Congo and Tanzania, respectively. The gene sequences from *Arvicanthis neumanni* were closely related to *Bartonella elizabethae*. The genotypes from *Lophuromys* spp. and from *Praomys delectorum* were close to *Bartonella tribocorum*. Five genogroups were not genetically related to any known *Bartonella* species. These results suggest the need to conduct further studies to establish the zoonotic risks linked with those *Bartonella* species and, in particular, to verify whether these agents might be responsible for human cases of febrile illness of unknown etiology in Africa.

INTRODUCTION

Bartonella species are zoonotic and vector-borne bacteria associated with an increasing array of emerging infections in humans and animals.[1–3] These bacteria are responsible for a wide range of clinical manifestations, including trench fever, cat-scratch disease, and endocarditis in immunocompetent patients, and bacillary angiomatosis and peliosis hepatitis in immunocompromised patients.[4] Bartonellae typically parasitize the erythrocytes of mammalian hosts, resulting in long-lasting infections. Several new *Bartonella* species have been isolated recently from a wide range of wild mammals, including rodents,[5–15] lagomorphs,[16,17] carnivores,[1,18] and ruminants.[1,19] The close association between rodents and humans throughout the world, especially in rural environments and in the overcrowded metropololis of sub-Saharan Africa, makes the study of rodent-borne *Bartonella* essential to determine the extent to which rodents may serve as a source of human infections.[20]

Bartonella species associated with small mammals have been detected in Asia, Australia, North America, and Europe.[11,15,20–27] Recent studies showed that bartonellae were widely distributed among rodents in South Africa[14] and among fleas in Democratic Republic of the Congo (DR Congo).[28] However, no study was conducted in small mammals of DR Congo or Tanzania. The aims of this study were 1) to investigate the prevalence of *Bartonella* infections in small mammal populations of selected areas of these two countries; 2) to evaluate the genetic diversity of *Bartonella* communities by analyzing partial sequences of *glt*A and *rpo*B genes; and 3) to compare *Bartonella* genotypes obtained from small animals in DR Congo and Tanzania with genotypes identified in Africa and other regions of the world.

MATERIALS AND METHODS

Mammal sampling. Small mammals were sampled either in crop fields or fallow land during March–April 2007 in the Rethy village of the Ituri district (N2.09176-E30.88982), DR Congo at elevations ranging from 1,960 to 2,120 m above sea level. In Mbulu district, northern Tanzania, rodents were trapped during February–March 2007 in two villages, namely Arri and Tumati, which are located in the Division of Dongobesh (S040 04-E0350 22) at altitudes ranging from 1,930 to 2,250 m a.s.l. Trapping was conducted in the forest near the hamlet of Mongahay in Tumati and in crop fields of Arri. Details of the captured animals are given in Tables 1 and 2.

Small mammals were captured mainly using Sherman traps (model LFA, 3 × 3, 5 × 9 in.; Sherman Traps Inc., Tallahassee, FL) baited with peanut butter mixed with maize flour. Occasionally, Tomahawk collapsible traps (model 202, Tomahawk Live Traps Co., WI), and locally made box-traps were also used. Rodents were trapped in various habitats including primary altitude natural forest, fallow land (shrubs and bushes), and crop fields. In Mbulu District, rodents were trapped in two principal habitats: 1) the natural rain forest with dense undergrowth and tall trees and 2) the crop field in the fringes of the forest. Trapping of the small mammals in these habitats was carried out for three consecutive nights with 100 Sherman traps per habitat per night. Each captured animal was transferred to the laboratory in a tissue bag and euthanized with ether. Tissue samples were taken from spleen and stored in 90% ethanol.

Each mammal was initially identified to genus level in the field. Before sample collection, the gender and species of animals were recorded. Species identification of the animals was confirmed in the laboratories of the University of Antwerp and the Royal Institute of Natural Science in Brussels (Belgium) by combining craniometrical measurements and mitochondrial DNA cytochrome-*b* sequencing.

Molecular screening for bartonellae DNA. Genomic DNA was isolated from spleen samples using tissue protocol of the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions and stored at –20°C. The quantity of DNA was measured using NanoDrop 2000 (Thermo Scientific, Wilmington, DE). Extracted DNA was used in all polymerase chain reaction (PCR) assays. Primers used were 1400F and 2300R[29] for an 825-bp specific fragment of *rpo*B gene.[29] For the specific fragments of the *glt*A, we used two combinations of the primers: CS140f-BhCS.1137n[30,31] and CS443f-BhCS.1137n[30] for 327 bp. The PCR was performed with 50-μL mixtures containing 20 ng of the DNA, 5 × Green

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TABLE 1
Prevalence of *Bartonella* in small mammals of Democratic Republic of Congo

Mammal species	Common name	Habitat	Area/district	No. positive/studied (% positive)	Total no. of positive/studied (% positive)
Arvicanthis neumanni	Neumann's grass rat	Crop field	Kpandruma	0/1 (0)	2/5 (40)
		–	Rethy	2/3 (66.7)	
		–	Zaa	0/1 (0)	
Crocidura sp.	White-toothed shrew	Swamp area	Djalusene	0/1 (0)	0/8 (0)
		–	Kpandruma	0/1 (0)	
		–	Rethy	0/3 (0)	
		–	Zaa	0/3 (0)	
Lophuromys rita	Yellow-spotted brush-furred rat	Crop field	Djalusene	0/1 (0)	1/4 (25)
		–	Rethy	1/3 (33.3)	
Mastomys coucha	Multimammate rat	Crop field	Kpandruma	0/4 (0)	0/10 (0)
		House	Zaa	0/6 (0)	
Mus minutoides	African pygmy mouse	Bushes	Djalusene	0/1 (0)	4/6 (66.7)
		Bushes	Rethy	3/4 (75)	
		Swamp area	Rethy	1/1 (100)	
Otomys sp.	African vlei rat	Crop filed	Rethy	0/1 (0)	0/1 (0)
Rattus rattus	Black rat	House, domestic environment	Djalusene	0/1 (0)	1/25 (4)
			Kpandruma	0/5 (0)	
			Rethy	0/2 (0)	
			Zaa	1/17 (5.9)	
Total (7 species)					8/59 (13.6)

GoTaq reaction buffer (10 μL), 200 μM of each dNTP, 1.25 U *Taq* DNA polymerase (Promega, Madison, WI), and 1.0 μM of each primer. Each PCR was conducted in a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA). The PCR was incubated at 94°C for 2 min to denature DNA and the thermal cycle reaction programmed for 38 cycles of 30 s at 94°C, 30 s at 52°C (for *rpo*B) and 48°C (for *glt*A), and 2 min at 72°C, with a 7-min final extension step at 72°C. The PCR products were subjected to electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. Amplicons of the expected size were identified by size comparison to the positive control. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Germantown, MD). Primers 1400F, 2028F, 1596R, 1873R, and 2300R for *rpo*B[29] and CS140f, CS443f, and BhCS.1137n for *glt*A[30] were used for DNA sequencing. Sequencing reactions were carried out with a PTC-200 Peltier Thermal Cycler, using Dye Terminator Cycle Sequencing with the Quick Start kit (Beckman Coulter, Fullerton, CA) using the following program: initial denaturing step for 1 min at 96°C, and 96°C for 10 s, 50°C for 5 s, 60°C for 4 min, and each step was repeated for 25 cycles. The DNA sequences were analyzed using Lasergene version 8 sequence analysis software (DNASTAR, Madison, WI). The SeqMan program (DNASTAR) was used to obtain consensus sequences for the amplified regions of the target genes. The DNA sequences of this study were deposited in GenBank (Table 3).

Phylogenetic analysis. Analysis of DNA sequences and phylogenetic relationships were done using MEGA4.[32] The DNA sequences of this study and the known *Bartonella* species retrieved from the GenBank were aligned using the Clustal X.[33] Phylogenetic trees were drawn separately based on the *rpo*B (825 bp) and *glt*A (327 bp) gene fragments, using the neighbor-joining method[34] with the Kimura 2-parameter distance model[35] in MEGA4.[32] The stability of inferred phylogenies was assessed by bootstrap analysis of 1,000 randomly generated sample trees.[36]

RESULTS

Animal collection. A total of 98 small mammals of 10 genera were used for this study (Tables 1 and 2). Fifty-nine animals, including 51 rodents of six genera, *Arvicanthis*, *Lophuromys*, *Mastomys*, *Mus*, *Otomys*, and *Rattus*, all belong to Muridae and 8 white-toothed shrews of the genus *Crocidura* of Soricidae, were sampled in DR Congo (Table 1). Thirty-nine rats of 4 genera (Muridae), *Grammomys*, *Lophuromys*, *Mus*, and *Praomys* were trapped in Tanzania (Table 2).

TABLE 2
Prevalence of *Bartonella* in rodents of Tanzania

Rodent species	Common name	Habitat	Area/district	No. of positive/studied (% positive)	Total no. of positive/studied (% positive)
Grammomys sp.	African thicket rat	Crop field and fallow land	Ari	1/1 (100)	1/1 (100)
Lophuromys sp. (*dudui*-related)	Brush-furred rat	Crop field and fallow land	Tumati	3/6 (50)	9/18 (50)
		Natural forest	Ari	0/1 (0)	
		–	Tumati	6/11 (54.5)	
Mus minutoides	African pigmy mouse	Crop field and fallow land	Ari	0/2 (0)	0/11 (0)
		–	Tumati	0/9 (0)	
Praomys delectorum	African soft-furred rat	Crop field and fallow land	Tumati	2/3 (66.7)	6/9 (66.7)
		Natural forest	–	4/6 (66.7)	
Total (4 species)					16/39 (41)

TABLE 3

*Bartonella rpo*B and *glt*A genotypes found in the small mammals of Democratic Republic of Congo and Tanzania

			rpoB genotyping				gltA genotyping			
Mammal species	Animal ID	Country	GenBank accession nos.	Genotype	Group/% similarity within group	Closest *Bartonella* spp./% similarity	GenBank accession nos.	Genotype	Group/% similarity within group	Closest *Bartonella* spp./ % similarity
Arvicanthis neumanni	An615drc	DR Congo	FJ851128	1-r	A-r	*B. elizabethae*/98.1	FJ851106	1-g	A-g/96–98.5	*B. elizabethae*/98.4
Praomys delectorum	Pd5696t	Tanzania	FJ851133	2-r	B-r	*B. elizabethae*/93.8	FJ851110	3-g	A-g	*B. elizabethae*/95.8
Lophuromys sp. (*dudui*-related)	Ld482t	Tanzania	FJ851146	3-r	C-r/96–99.6	*B. tribocorum*/93.9	FJ851122	5-g	C-g/96.4–99.7	*B. elizabethae*/96
Lophuromys sp. (*dudui*-related)	Ld481t	Tanzania	FJ851144	4-r	C-r	*B. tribocorum*/93.8	FJ851121	6-g	C-g	*B. elizabethae*/96
Lophuromys rita	Lr601drc	DR Congo	FJ851123	5-r	C-r	*B. tribocorum*/94.1	FJ851103	16-g	G-g	*B. birtlesii*/91.1
Lophuromys sp. (*dudui*-related)	Ld5743t	Tanzania	FJ851132	6-r	C-r	*B. tribocorum*/93.6	FJ851109	7-g	C-g	*B. elizabethae*/96.3
Lophuromys sp. (*dudui*-related)	Ld5707t	Tanzania	FJ851136	6-r	C-r	*B. tribocorum*/93.6	FJ851113	7-g	C-g	*B. elizabethae*/96.3
Lophuromys sp. (*dudui*-related)	Ld5706t	Tanzania	FJ851140	6-r	C-r	*B. tribocorum*/93.6	FJ851117	7-g	C-g	*B. elizabethae*/96.3
Lophuromys sp. (*dudui*-related)	Ld5742t	Tanzania	FJ851134	7-r	C-r	*B. tribocorum*/93.6	FJ851111	15-g	G-g	*B. birtlesii*/92.6
Mus minutoides	Mm625drc	DR Congo	FJ851125	8-r	C-r	*B. tribocorum*/94.3	FJ851104	4-g	B-g	*B. elizabethae*/93.6
Mus minutoides	Mm627drc	DR Congo	FJ851126	8-r	C-r	*B. tribocorum*/94.3	FJ851105	4-g	B-g	*B. elizabethae*/93.6
Mus minutoides	Mm628drc	DR Congo	FJ851129	8-r	C-r	*B. tribocorum*/94.3	FJ851107	4-g	B-g	*B. elizabethae*/93.6
Mus minutoides	Mm604drc	DR Congo	FJ851124	8-r	C-r	*B. tribocorum*/94.3	nd			
Lophuromys sp. (*dudui*-related)	Ld480t	Tanzania	FJ851143	9-r	C-r	*B. tribocorum*/94.8	FJ851120	8-g	D-g	*B. queenslandensis*/95.4
Grammomys sp.	Gs5686t	Tanzania	FJ851135	10-r	D-r	*B. grahamii*/94.1	FJ851112	9-g	E-g	*B. queenslandensis*/95.7
Arvicanthis neumanni	An616drc	DR Congo	FJ851127	11-r	E-r	*B. grahamii*/92.8	nd			
Praomys delectorum	Pd5700t	Tanzania	FJ851138	12-r	F-r/97.9–99.9	*B. grahamii*/92.5	FJ851115	2-g	A-g	*B. elizabethae*/97.1
Praomys delectorum	Pd5695t	Tanzania	FJ851137	13-r	F-r	*B. grahamii*/92.4	FJ851114	10-g	F-g/96.3–99.7	*B. tribocorum*/97.9
Praomys delectorum	Pd5708t	Tanzania	FJ851139	14-r	F-r	*B. grahamii*/92.2	FJ851116	11-g	F-g	*B. tribocorum*/97.5
Praomys delectorum	Pd5728t	Tanzania	FJ851142	14-r	F-r	*B. grahamii*/92.2	FJ851119	12-g	F-g	*B. tribocorum*/96.6
Rattus rattus	Rr641drc	DR Congo	FJ851130	15-r	F-r	*B. grahamii*/92.6	nd			
Praomys delectorum	Pd5692t	Tanzania	FJ851131	16-r	F-r	*B. grahamii*/92.4	FJ851108	13-g	F-g	*B. tribocorum*/96.3
Lophuromys sp. (*dudui*-related)	Ld479t	Tanzania	FJ851145	17-r	F-r	*B. grahamii*/92.4	nd			
Lophuromys sp. (*dudui*-related)	Ld5693t	Tanzania	FJ851141	18-r	G-r	*B. birtlesii*/91.2	FJ851118	14-g	G-g/96.0–98.2	*B. birtlesii*/92.9

nd = not detected.

All field procedures involving animal sample collection were conducted under protocols approved by the University of Antwerp.

Prevalence of *Bartonella* in DR Congo and Tanzania. By *rpo*B-PCR, *Bartonella* DNA was detected in 8 of 59 (13.6%) rodents sampled in DR Congo: in 2 *Arvicanthis neumanni*, 1 *Lophuromys rita*, 4 *Mus minutoides*, and 1 *Rattus rattus* (Table 1). The highest prevalence was observed in *M. minutoides*, 4 of 6 (67.0%). The lowest rate of *Bartonella* infection was found in *R. rattus*, 1 of 25 (4.0%). By *glt*A-PCR, *Bartonella* DNA was detected in 5 (8.5%) animals collected in DR Congo. The *glt*A-PCR was negative in one of *A. neumanni* and *M. minutoides* and in *R. rattus* animals, which were positive by the *rpo*B-PCR. No evidence of infection was found in 19 animals: *Crocidura* sp. ($N = 8$), *Mastomys coucha* ($N = 10$), and *Otomys* sp. ($N = 1$).

In Tanzanian rodents, 1 *Grammomys* sp. (100%), 9 *Lophuromys* sp. (50%), and 6 *Praomys delectorum* (66.7%) were infected by *Bartonella* (Table 2). The *rpo*B sequences were obtained in 16 out of a total 39 animals (41.0%). By *glt*A-PCR, *Bartonella* DNA was detected in 15 (38.5%) animals of Tanzania. The *glt*A-PCR was negative in one of *Lophuromys* sp., in which *Bartonella* DNA was detected by the *rpo*B gene. No evidence of infection was found in 9 *M. minutoides*.

Genetic diversity of *Bartonella* DNA sequences. In this study, *rpo*B (825 bp) and *glt*A (327 bp) sequences were obtained from 24 and 20 animals, respectively (Table 3). Comparison of the 24 *rpo*B gene sequences revealed 18 genotypes (Table 3). The sequence homology ranging from 88.2% to 99.9% was found among *rpo*B genotypes. Among 20 *glt*A sequences, we found 16 genotypes with 87.4–99.7% homology (Table 3). All genotypes were further categorized into seven groups, A-r to G-r for *rpo*B and A-g to G-g for *glt*A, based on sequence similarities and a clustered pattern in phylogeny (Figures 1 and 2; Table 3). In this study, some *Bartonella* groups appear to follow a specific pattern that reflects host specificity. This specific pattern was clearly observed especially in the *glt*A phylogeny (Figure 2). For example, the *glt*A genotypes of groups B-g, C-g, F-g, and G-g identified in *Mus*, *Lophuromys*, *Praomys*, and *Lophuromys* animals, respectively, were clustered together in different branches in the phylogeny (Figure 2). In this study, several groups were close to two well-known rat-associated bartonellae: *rpo*B groups A-r, B-r, and *glt*A groups A-g, B-g, C-g, for *B. elizabethae; rpo*B group C-r and *glt*A group F-g for *Bartonella tribocorum.* However, only the genotype 1-r of *rpo*B and 1-g of *glt*A were concordant in both phylogenies and very close to *Bartonella elizabethae* with respective similarities 98.1% and 98.4%, which were higher than the cut-off values 95.4% and 96% for *rpo*B and *glt*A genes, respectively, according to La Scola and others.[37] Similarly, *rpo*B 18-r of G-r group was concordant with *glt*A 14-g of G-g group and clustered with *Bartonella birtlesii* in both phylogenies. In contrast, other genotypes were not concordant with both phylogenies. For example, the genotypes of *rpo*B groups C-g to F-g were close to *B. tribocorum* and *Bartonella grahamii*; however, counterparts of the *glt*A groups from the same animals were close to *B. elizabethae*, *B. birtlesii*, *Bartonella queenslandensis*, and *B. tribocorum*. Overall, several genotypes found in the animals were not closely related to any described *Bartonella* species and were mostly belonging to unknown species, as per cut-off values determined by La Scola and others.[37]

DISCUSSION

Our study reports the first detection and prevalence of *Bartonella* species in rodent populations from DR Congo and Tanzania. To our knowledge, it is also the first report of detection of *Bartonella* spp. in several rodent species: *A. neumanni*, *L. rita*, and *M. minutoides*, *Grammomys* sp., *Lophuromys* sp., and *P. delectorum*. The overall prevalence of *Bartonella* in six species (*A. neumanni, L. rita*, *M. minutoides* from DR Congo; *Grammomys* sp., *Lophuromys* sp., and *P. delectorum* from Tanzania) of the 11 examined rodent species was remarkably high (25–100%), although it was 4% in *R. rattus*. However, the number of animals trapped for this study was very low for four rodent species, which could explain our failure to identify *Bartonella* in these animals (Tables 1 and 2).

The prevalence and diversity of *Bartonella* species was reported previously in 10 species of small mammals collected in Free State province of South Africa.[14] In this study, 9 *Mastomys natalensis* ($N = 15$), 1 *Otomys irroratus* ($N = 2$) were positive, and the only *R. rattus* examined was not infected by *Bartonella*.[14] On the contrary, in our study, 10 and 1 specimen of related species, *Mastomys coucha* and *Otomys* sp., respectively, were found to be negative and 1 *R. rattus* was positive for *Bartonella*. A total of 24 out of 98 animals were infected by *Bartonella* species in our study. This prevalence was comparable to previous reports from other countries; ranging from 9% to 44% in Asia, 17% to 64% in Europe, 42% in North America, 44% in South Africa, and 29% in Australia.[5,8,9,11,13–15,20,22,24,38–41] Such high rates of *Bartonella* prevalence could be significant with respect to the risks of humans becoming infected with these agents. The commensal mammals harboring pathogenic microorganisms are often found in biotopes where they can come into close contact with humans who might therefore be at some risk of exposure. In this study, considerably high levels of heterogeneities were found among *rpo*B and *glt*A gene sequences. Moreover, several *rpo*B genotypes were not concordant with *glt*A genotypes sequenced in the same animal, and hence, a similar branching pattern was not observed between two phylogenetic trees. This genotypic heterogeneity might be caused by the environmental conditions, host animal and its specificity, ectoparasites, etc., in the respective geographical locations.

In this study, *glt*A genotypes of genogroups B-g, C-g, and F-g obtained, respectively, from *M. minutoides*, *Lophuromys* sp., and *P. delectorum* were very close to each other in their respective groups and clustered together as separate branches (Figure 2). This finding suggests the host specificity between the genotypes in these genogroups and the host. Moreover, this host specificity was also observed in the *rpo*B genotyping (Figure 1). According to Ellis and others,[21] *Bartonella* associated with hosts native to the Old World are phylogenetically distinct from those associated with a host-specific native to the New World. Several genotypes of this study were completely novel and had no evolutionary relationships with other known *Bartonella*, which supports the hypothesis of Ellis and others. However, some genotypes from Congolese and Tanzanian small mammals in the phylogenetic trees clustered with well-known rodent-associated species of bartonellae, including *B. elizabethae*, *B. tribocorum,* and *B. queenslandensis* (Figures 1 and 2). In this context, further studies should be conducted on a large collection of rodents and small animals from Africa to determine the

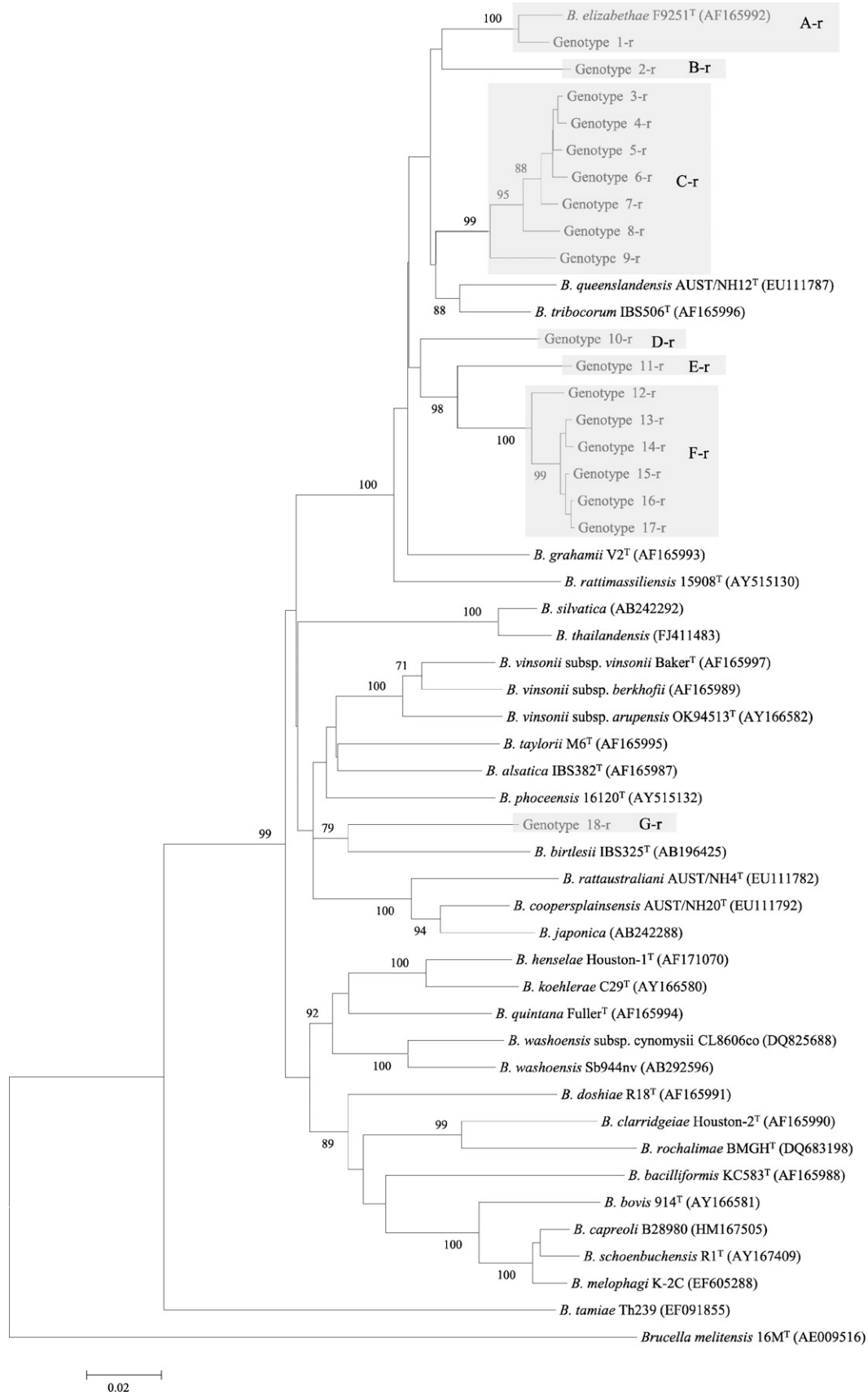

FIGURE 1. Phylogenetic classification of *Bartonella* genotypes based on *rpo*B gene sequences. Only the bootstrap values above 70% obtained are given. The GenBank accession numbers for reference sequences are given in parentheses. The *rpo*B gene sequences of *Brucella melitensis* 16M^T were included as an out-group.

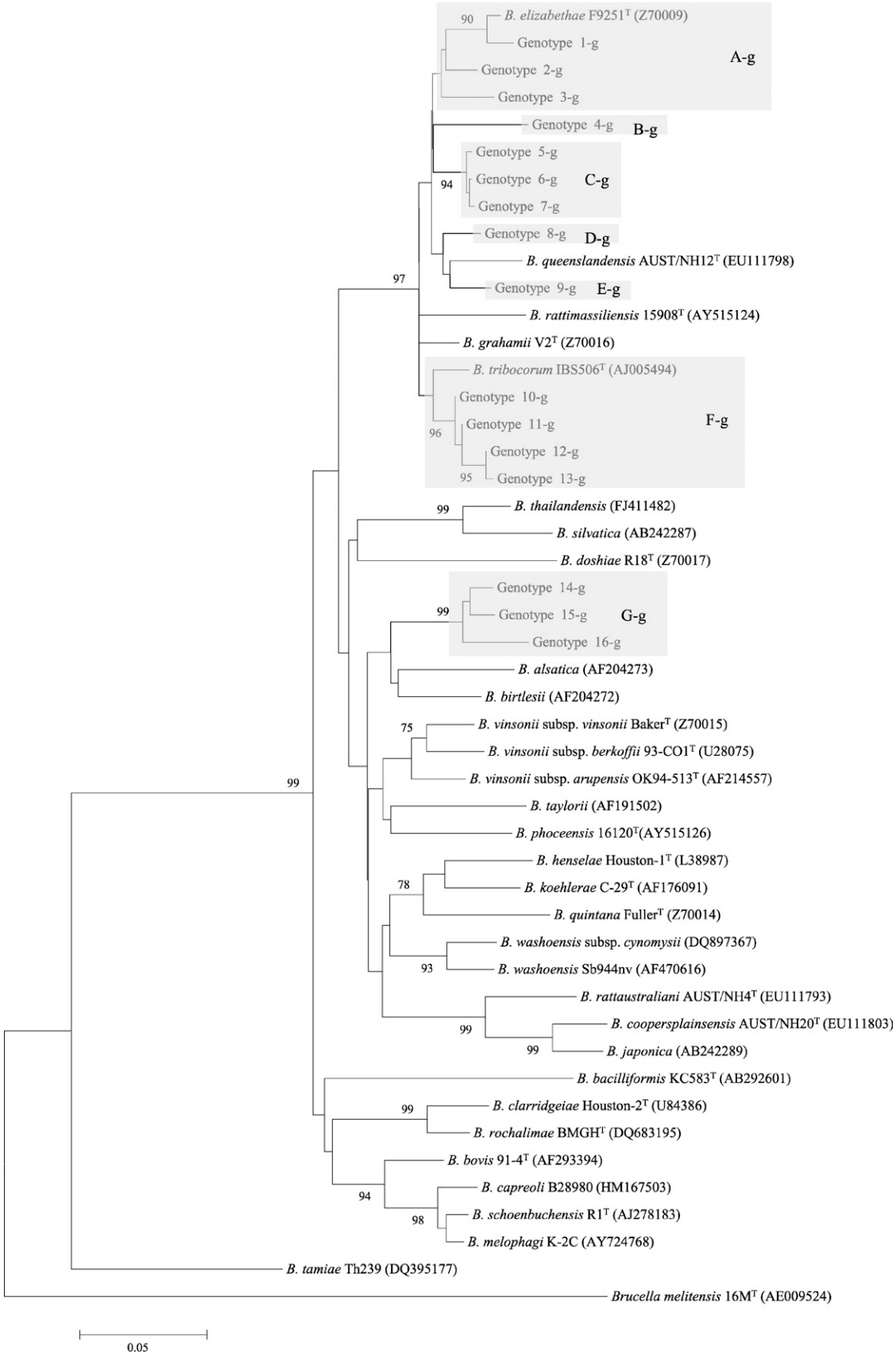

FIGURE 2. Phylogenetic classification of *Bartonella* genotypes based on *glt*A gene sequences. Only the bootstrap values above 70% obtained are given. The GenBank accession numbers for reference sequences are given in parentheses. The *glt*A gene sequences of *Brucella melitensis* $16M^T$ were included as an out-group.

evolutionary, genetic, and pathogenic relationships between African and other isolates.

The results suggest the need to conduct further studies to verify whether these agents might be responsible for human cases of febrile illness of unknown etiology in these countries. These preliminary data will allow us to design further studies on the comprehensive survey of the risks associated with exposure to rodent-associated *Bartonella* in these countries and other regions in Africa. In this context, future studies will be concentrated on isolation of bartonellae from African small animals.

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Disclosure: Dr. Gundi was an EID Fellow during 2008–2009. He is working in Bartonella Laboratory, Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado. His research interests include tropical and emerging infectious diseases.

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