

## Isolation of *Bartonella henselae*, *Bartonella koehlerae* subsp. *koehlerae*, *Bartonella koehlerae* subsp. *bothieri* and a new subspecies of *B. koehlerae* from free-ranging lions (*Panthera leo*) from South Africa, cheetahs (*Acinonyx jubatus*) from Namibia and captive cheetahs from California

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Received 18 March 2016; Final revision 23 May 2016; Accepted 8 June 2016;  
first published online 25 July 2016

### SUMMARY

Bartonellae are blood- and vector-borne Gram-negative bacteria, recognized as emerging pathogens. Whole-blood samples were collected from 58 free-ranging lions (*Panthera leo*) in South Africa and 17 cheetahs (*Acinonyx jubatus*) from Namibia. Blood samples were also collected from 11 cheetahs (more than once for some of them) at the San Diego Wildlife Safari Park. Bacteria were isolated from the blood of three (5%) lions, one (6%) Namibian cheetah and eight (73%) cheetahs from California. The lion *Bartonella* isolates were identified as *B. henselae* (two isolates) and *B. koehlerae* subsp. *koehlerae*. The Namibian cheetah strain was close but distinct from isolates from North American wild felids and clustered between *B. henselae* and *B. koehlerae*. It should be considered as a new subspecies of *B. koehlerae*. All the Californian semi-captive cheetah isolates were different from *B. henselae* or *B. koehlerae* subsp. *koehlerae* and from the Namibian cheetah isolate. They were also distinct from the strains isolated from Californian mountain lions (*Felis concolor*) and clustered with strains of *B. koehlerae* subsp. *bothieri* isolated from free-ranging bobcats (*Lynx rufus*) in California. Therefore, it is likely that these captive cheetahs became infected by an indigenous strain for which bobcats are the natural reservoir.

**Key words:** *Acinonyx jubatus*, *Bartonella henselae*, *Bartonella koehlerae*, cheetah, lion, *Panthera leo*.

### INTRODUCTION

Members of the genus *Bartonella* are aerobic Gram-negative bacteria which are mainly vector-

borne and recognized as emerging pathogens [1]. Many new species or subspecies have been described in recent years and the genus currently consists of at least 30 species, 16 of which are associated with human diseases [2]. The clinical spectrum of symptoms in humans caused by *Bartonella* species is considered to be widening [1]. The most common infection is cat scratch disease, caused by *B. henselae*, with domestic cats (*Felis catus*) being the natural

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reservoir [3–5]. Domestic cats are also the natural reservoir for *B. clarridgeiae* and *B. koehlerae* [6–8].

Free-ranging wild felids, just like domestic cats, can be infected with *Bartonella* species [9–11]. In North America, free-ranging mountain lions and bobcats are infected with *Bartonella* species similar to but different from *B. henselae* and *B. koehlerae*, proposed as *B. koehlerae* subsp. *boulouisii* and *B. koehlerae* subsp. *bothieri* [11, 12], and also infected with *B. henselae* [12, 13]. In Africa, *B. henselae* was isolated from free-ranging lions (*Panthera leo*) from Kruger Park, South Africa [10]. It was also isolated from semi-captive lions from three ranches in the Free State Province, South Africa [14] and Kelly *et al.* [15] had reported the isolation of *B. henselae* from a pet cheetah in Zimbabwe. A *ftsZ* gene sequence close (99%) to *B. koehlerae* has also been detected by polymerase chain reaction (PCR) in a captive margay cat (*Leopardus wiedii*) born in the wild and kept at Sao Paulo Zoo [16].

As we have recently described new subspecies of *B. koehlerae* in wild felids from North America [12], the objective of this study aimed to fully describe the *Bartonella* species previously isolated from free-ranging cheetahs from Namibia and lions from Kruger Park, South Africa [10] in order to determine if they belonged to a new *Bartonella* species or subspecies, as well as *Bartonella* isolated from captive cheetahs at the San Diego Zoo Safari Park, CA, USA.

## MATERIALS AND METHODS

### Bacterial strains

*B. henselae* type I (Houston 1) ATCC 49882 and *B. clarridgeiae* ATCC 51734 were obtained from the American Type Culture Collection (Rockville, MD). Strain U4 originally isolated from a naturally infected cat at the University of California Davis was used for the *B. henselae* type II (Marseille type). *B. koehlerae* strain C29 (ATCC 700693) was isolated from a naturally infected barn cat [7]. *Candidatus B. weissii* (now *B. bovis*), isolated from domestic cats and the *B. henselae* Zimbabwean cheetah strain were kindly provided by Russell L. Regnery (Centers for Disease Control and Prevention, USA).

### Clinical samples

*Animals.* Whole blood samples (1.5–2.0 ml per tube) were collected in EDTA plastic tubes (Becton

Dickinson and Company, USA) from 58 free-ranging lions at Kruger Park, South Africa and 17 cheetahs held in a rehabilitation centre after being rescued from various regions of Namibia, as previously reported [10]. At the San Diego Zoo Safari Park, blood samples were collected from 11 cheetahs in 1999 (Table 1), of which four had blood collected more than once. These animals were kept in a large open enclosure during the day. Collection of blood samples by veterinarians was performed under animal use and care protocols of the San Diego Zoo Safari Park and at the Cheetah Conservation Fund facility. Animals were tranquillized using 11 mg/kg ketamine hydrochloride via the intramuscular route. All blood samples were stored at –20 °C or lower until they were processed for culture.

### Blood culture

In 2003, the whole-blood samples were defrosted and then centrifuged at 5000 *g* for 30 min at room temperature. Blood pellets were resuspended in 125  $\mu$ l M199 inoculation medium [17] and plated onto heart infusion agar (Difco Laboratories, USA) containing 5% fresh rabbit blood. The plates were then incubated at 35 °C in 5% CO<sub>2</sub> for 4 weeks, and cultures were examined at least twice a week for bacterial growth. The number of colonies observed was recorded as the number of colony-forming units per milliliter (c.f.u./ml) of blood. Colonies were subcultured, harvested, and frozen at –70 °C.

### Microscopic analysis

Gram staining was performed on all isolates. Sterile swabs moistened with filter-sterilized phosphate-buffered saline (PBS) were used to remove colonies from subcultured agar plates to glass slides. Following heat fixation, Gram staining was used for light microscopic visualization of organisms. The motility of cells suspended in heart infusion broth was determined with a 100 $\times$  oil immersion objective.

### Indirect immunofluorescence antibody (IFA) test

Antibody titres against *B. henselae* were determined using an IFA test [10]. For antigen preparation, *B. henselae* (strain U4) was cultivated on Vero cells for 3–5 days. Infected Vero cells were then suspended in L15/MEM tissue culture medium (Invitrogen, USA). Infected Vero cells were applied to each well

Table 1. *Bartonella* bacteraemia level, serological titres, sex, age and date of collection of blood samples from 11 semi-captive cheetahs (*Acinonyx jubatus*), San Diego Zoo Safari Park, California

ID	Sex	Age, yr	Date collected	c.f.u./ml	IFA ( <i>B.h.</i> )
MO692327	Male	6	21 Jan. 1999	22	0
MO692326	Male	6	27 Jan. 1999	35	128
MO692328	Male	6	15 Jan. 1999	5	0
MO691036	Female	8	26 Feb. 1999	355	0
			24 June 1999	20	0
MO689192	Female	10	21 Jan. 1999	250	32
			25 May 1999	1322	64
MO692324	Female	7	9 June 1999	0	0
			5 July 1999	0	0
MO695628	Female	4	14 June 1999	373	64
			18 Oct. 1999	168	64
MO692586	Male	6	22 June 1999	0	128
MO691033	Male	8	10 Aug. 1999	0	0
MO691023	Male	8	27 Aug. 1999	10	±32
MO689255	Male	10	14 Sept. 1999	1043	0

*B.h.*, *Bartonella henselae*; c.f.u./ml, colony-forming units/millilitre; IFA, immunofluorescence assay.

of multi-well super-cured heavy Teflon-coated slides (Erie Scientific Co., USA) and incubated for 24 h to allow the cells to adhere to the slides. Slides were then rinsed in PBS (pH 7.4), air-dried, acetone-fixed, and stored at  $-20^{\circ}\text{C}$  after a final air-drying. Wild cat sera (supernatant from the whole-blood samples) were diluted 1:64 in PBS with 5% skim milk, and added to the test wells of the slides. After washing in PBS, fluorescein-labelled goat anti-cat IgG (Cappel, Organon Teknika Corp., USA) was used as the conjugate. Positive and negative controls were included on each test slide. Intensity of fluorescence of the bacteria was graded subjectively on a scale of 0–4. Fluorescence intensity  $\geq 2$  at a dilution of 1:64 was considered to be a qualitatively positive result, as described previously [9]. For a quantitative result, positive sera were serially diluted (twofold dilutions) to obtain an endpoint titre. Reading of the slides was performed independently by two readers for all serum samples.

#### DNA extraction and PCR

Subcultured colonies were scraped off and suspended in 100  $\mu\text{l}$  sterile water. The bacterial suspension was heated for 15 min at  $100^{\circ}\text{C}$  and centrifuged at 15 000 *g* for 10 min at  $4^{\circ}\text{C}$ . The supernatant, diluted 1:10, was then used as a template for amplification of the 16S rRNA, *gltA*, *ribC*, *groEL* and *ftsZ* genes as well as the 16S-23S intergenic spacer region (ITS). About 380 base pairs (bp) of the *gltA* gene [18],

1500 bp of the 16S rRNA gene [19], 1500 bp of the *groEL* gene [20], 700 bp of 16S-23S ITS [21], 764 bp of the *ftsZ* gene [22], 586 bp of the *ribC* gene [23] and fragments of various size of 16S-23S ITS [24] were amplified using previously described primers and methods and verified by gel electrophoresis.

#### Restriction fragment length polymorphism (RFLP)

The amplified products were enzymatically digested overnight using various restriction endonucleases. The amplified product of the *gltA* gene was digested with *TaqI* (Promega, USA), *HhaI*, *MseI*, and *AciI* (New England Biolabs, USA), the amplified product of the 16S rRNA gene was digested with *DdeI* (New England Biolabs), the amplified product of the *ribC* gene was digested with *TaqI*, and the amplified product of the 16S-23S ITS gene was digested with *TaqI* and *HaeIII*. The temperature of digestion was  $65^{\circ}\text{C}$  for *TaqI* and  $37^{\circ}\text{C}$  for all other enzymes. The digested fragments were separated by electrophoresis in a 3% Nusieve GTG agarose gel (Biowhittaker Molecular Applications, USA). Fragment sizes were estimated by comparison with a 100-bp ladder (Invitrogen).

#### Sequencing

PCR products used for DNA sequencing were purified with QIAquick PCR purification kit (Qiagen Sciences, USA) and sequencing was done in both directions using a fluorescence-based automated sequencing

system (Davis Sequencing, USA). For the phylogenetic analysis, sequence data of the *gltA*, *ftsZ*, *rpoB* [25] genes and the ITS region were imported into Vector NTI Suite 9.0 software (Invitrogen) in order to obtain a consensus sequence. Align X in Vector NTI was utilized for aligning sequence variants with each other and other known species of *Bartonella* for each of the genes or ITS. A neighbour-joining tree was constructed using MEGA v. 5.0 (<http://www.megasoftware.net>) by concatenating the four sequences [26]. Bootstrap replicates were performed to estimate node reliability of the phylogenetic tree, with values obtained from 1000 randomly selected samples of the aligned sequence data. The evolutionary distances were computed using the Kimura two-parameter method [27].

## RESULTS

### Primary isolation

In primary isolation, very small colonies were observed growing on 5% rabbit blood heart infusion agar plates after at least 14 days following plating of the blood pellets from three (5.2%) of the 58 lions, one (5.9%) of the 17 Namibian cheetahs, as previously reported [10] and eight (73%) of the 11 semi-captive cheetahs from California (Table 1). All bacterial colonies were homogeneous, either rough or smooth, round, grey-white in colour, 0.3–1.0 mm in diameter and embedded in the medium. The numbers of c.f.u./ml for the lion's isolates were respectively 35 and 93 c.f.u./ml in two adult females and 2000 c.f.u./ml in a juvenile male, as previously reported [10]. For the adult male cheetah that was culture positive, the number of colonies was 49 c.f.u./ml. For the Californian semi-captive cheetahs, bacteraemia ranged between 5 and 1322 c.f.u./ml (Table 1).

### Microscopic examination.

Microscopic examination after Gram staining revealed short, slender Gram-negative rods. No motility or flagella were detected.

### Serology

Serological results for the African free-ranging wild felids have been presented previously [10]. For the semi-captive cheetahs from California, five (45.5%) animals were seropositive with titres ranging from 1:64 to 1:128. Two cheetahs had low titres ( $\leq 64$ ) and five culture-positive cheetahs were seronegative.

### PCR/RFLP analysis

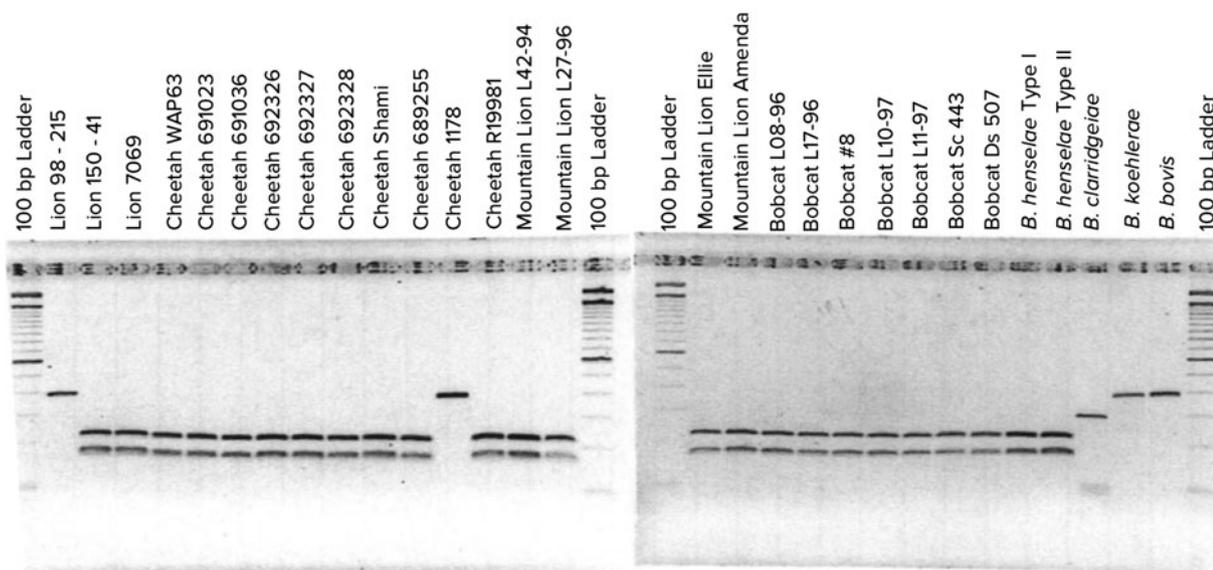
Two of the three lion isolates had profiles similar to *B. henselae* for the *gltA* gene when using *TaqI*, *HhaI* and *AcI*I enzymes and the ITS gene using *TaqI*. The PCR/RFLP profiles after digestion of the *gltA* gene by *TaqI* and *HhaI* endonucleases showed that the Namibian cheetah strain (1178) and the lion strain 98–215 were similar to *B. koehlerae* subsp. *koehlerae*'s profile with only lion strain 98–215 having a profile similar to *B. koehlerae* subsp. *koehlerae* after digestion with *AcI*I. A unique profile for cheetah strain 1178 and lion strain 98–215 was obtained for the *gltA* gene using *HhaI* endonuclease (Fig. 1), and for the *ribC* gene using *TaqI* endonuclease (data not shown), different from all other wild cat strains, but similar to *B. koehlerae* and *B. bovis* (ex *weissii*). Using the *DdeI* enzyme for the 16S rRNA gene, all isolates were determined to have the same profile as *B. henselae* type II, with the exception of the lion isolate 98–215 which was similar to the profiles of *B. henselae* type I, *B. koehlerae* subsp. *koehlerae* and *B. bovis* (formerly *B. weissii*).

### Sequencing

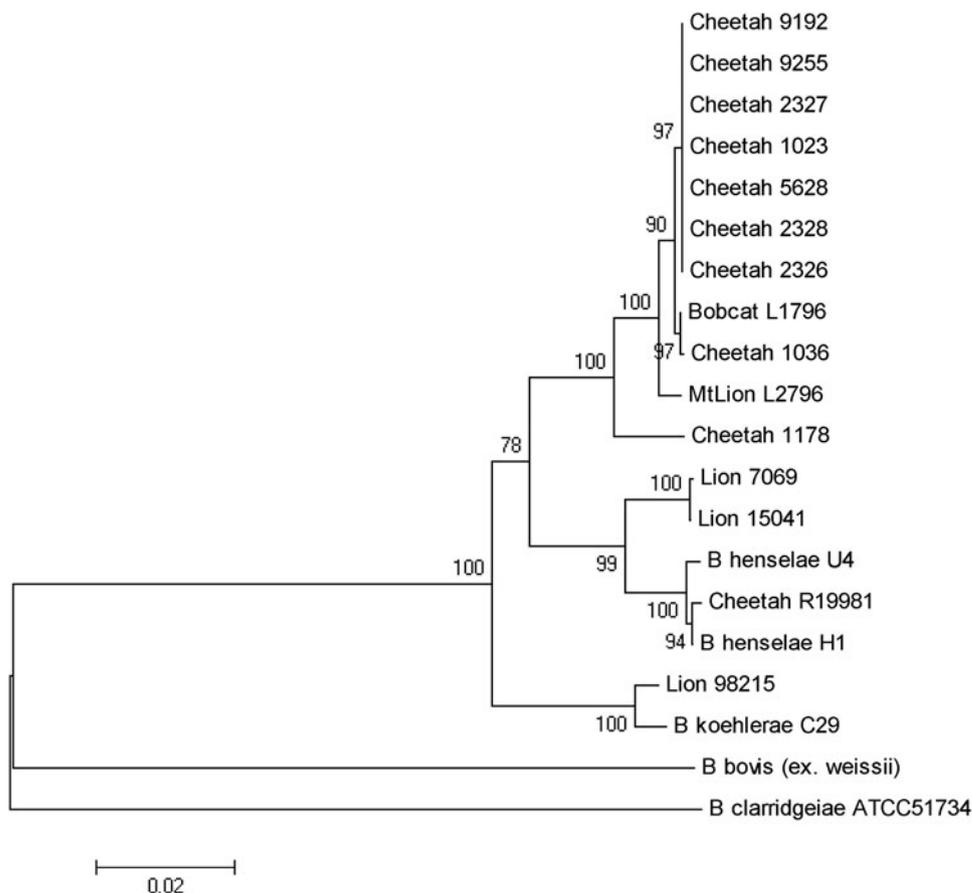
As previously reported, *B. henselae* was isolated from the blood of two of the bacteraemic lions (isolates 150-41 and 7069). Both isolates were identified as *B. henselae* type II (Marseille) based on partial sequencing of the 16S RNA gene. The third isolate from a juvenile (aged <24 months) male lion (98-215) was determined to be 100% identical to *B. koehlerae* subsp. *koehlerae* by partial sequencing of three different genes (*gltA*, *ftsZ*, *rpoB*) and the 16S-23S ITS, as shown for the concatenated tree (Fig. 2). The three bacteraemic lions were from different locations in Kruger National Park, South Africa. The *Bartonella* strain isolated from the blood of the bacteraemic cheetah from Gobabis District, Namibia was a previously unidentified strain temporarily designated as 'Cheetah 1178'. The California captive cheetahs all had an identical PCR/RFLP profile and partial sequencing revealed that they were identical to *B. koehlerae* subsp. *bothieri* (strain L1796), isolated from a California bobcat [12].

## DISCUSSION

*B. henselae* was isolated from two adult lions, as reported previously [10]. Both isolates were identified as type II (Marseille) based on partial sequencing of



**Fig. 1.** PCR/RFLP of the *gltA* gene using *HhaI* endonuclease, showing a unique profile for cheetah strain 1178 and lion strain 98–215 compared to all other wild felid isolates (no digestion), similar to *B. koehlerae* and *B. bovis* (ex *weissii*).



**Fig. 2.** Phylogenetic tree of *Bartonella* species based on the combined *gltA*, *rpoB*, *ftsZ*, and intergenic transcribed spacer sequence alignment. The tree shown is a neighbour-joining tree based on the Kimura two-parameter model of nucleotide substitution. Bootstrap values are based on 1000 replicates. The analysis provided tree topology only; the lengths of the vertical and horizontal lines are not significant.

the 16s RNA gene. An isolate of *B. henselae* type II was also reported from a lion living in one of three game farms in the Free State, South Africa [14]. The third isolate from a young male lion was determined to be 100% identical to *B. koehlerae* subsp. *koehlerae* by PCR/RFLP and by partial sequencing of two different genes (*gltA* *ftsZ*) and the 16S-23S ITS. This is the first report of isolation of *B. koehlerae* subsp. *koehlerae* from a free-ranging wild felid and expands the spectrum of felids that can be naturally infected with this *Bartonella* species. Until that isolate, *B. koehlerae* subsp. *koehlerae* had only been isolated from domestic cats in the United States, France and Israel [7, 28–30] and *B. koehlerae* subsp. *koehlerae* DNA had been detected from cat and rodent fleas [28, 31], a captive margay cat [16] as well as from three of 61 dogs that were culture- or PCR-positive for *Bartonella* species after enrichment [32]. It was also identified in two cases (a human and a dog) of endocarditis [29, 33]. This study confirms that besides domestic cats, free-ranging wild felids are infected and could be reservoirs of *B. henselae*, the agent of cat scratch disease, and *B. koehlerae* subsp. *koehlerae*, a known human pathogen.

Kelly *et al.* [15] reported the isolation of *B. henselae* genotype II from a pet cheetah from Zimbabwe. The present study is the first to report the isolation of a *Bartonella* species from a wild-caught cheetah. The PCR/RFLP profiles, using various endonucleases, led to a specific profile (no digestion) for cheetah strain 1178 and lion strain 98–215 for the *gltA* gene using *HhaI* endonuclease and for the *ribC* gene using *TaqI* endonuclease. This was different from all other wild cat strains but similar to *B. koehlerae* and *B. bovis*. Furthermore, partial sequencing of *gltA*, *groEL*, *ftsZ* genes and ITS put this Namibian cheetah strain (1178) in an intermediate position between *B. henselae* and *B. koehlerae*, as shown for strains isolated from free-ranging mountain lions and bobcats from California [12], but clearly distinct from the cheetah isolates from the San Diego Zoo.

The high infection rate of the semi-captive cheetahs from southern California was unexpected, but could be explained by the frequent contacts between these animals and the sharing of ectoparasites such as fleas. Interestingly, all the cheetah isolates were different from *B. henselae* or *B. koehlerae* subsp. *koehlerae* isolates, and from the Namibian cheetah isolate, clustering with the *B. koehlerae* subsp. *bothieri* strains isolated from free-ranging bobcats (*Lynx rufus*) in California. They also were distinct from the *B. koehlerae* subsp. *boulouisii* strain isolates from mountain

lions (*Felis concolor*) from California. Therefore, it is very likely that the semi-captive cheetahs became infected by an indigenous strain for which bobcats are the natural reservoir, possibly through infected fleas carried by bacteraemic bobcats.

The strains isolated from mountain lions, bobcats and cheetahs were morphologically similar to *B. henselae* and *B. koehlerae*, but they were not noticeable on blood agar for 2 weeks after plating. By contrast, *Bartonella* colonies are usually visible within a few days for domestic cat isolates. This characteristic was also observed in specific pathogen-free domestic kittens experimentally infected with one of the mountain lion isolates [8].

## ACKNOWLEDGEMENTS

The authors thank Russell L. Regnery for providing the cheetah strain from Zimbabwe. This project has been funded in part by the George and Phyllis Miller Feline Research Fund, Center for Companion Animal Health, U.C. Davis, California, the Master of Preventive Veterinary Medicine Research Project Fund (University of California, Davis) and Merial Inc., Athens, GA. Sophie Molia was a recipient of a Lavoisier grant (French Ministry of Foreign Affairs) and Barron fellowship (University of California, Davis). Jane E. Koehler received funding support from a California HIV Research Program Award, and NIH grants U54AI065359 and R01AI103299 from the NIAID.

## DECLARATION OF INTEREST

None.

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