Molecular Detection of *Bartonella henselae* DNA in the Dental Pulp of 800-Year-Old French Cats

Vu Dang La,[1] Benoît Clavel,[2] Sébastien Lepetz,[2] Gérard Aboudharam,[1] Didier Raoult,[1] and Michel Drancourt[1]

[1]Unité des Rickettsies, Faculté de Médecine, Université de la Méditerranée, Marseille, and [2]Laboratoire d'Archéozoologie de Compiègne, Centre de Recherche Archéologique de la Vallée de l'Oise, Compiègne, France

***Bartonella* species are responsible for chronic bacteremia in domestic cats, which raises a question about the antiquity of the relationship between *Bartonella* species and cats that act as reservoirs for the organism. The sequencing of *Bartonella pap31* and *groEL* genes from the dental pulp of cats dating from the 13th to 16th centuries identified the presence of *B. henselae* genotype Houston; the observation of a unique mutation in the results of PCR assays for *Bartonella* species ruled out modern DNA contamination of the dental pulp samples. We conclude that cats had bacteremia due to *B. henselae* 800 years ago.**

The genus *Bartonella* comprises facultative, intracellular, fastidious bacilli of the α2 subclass of Proteobacteria. Common features of *Bartonella* species include transmission by an arthropod vector and survival within mammalian hosts that act as reservoirs [1]. Four *Bartonella* species have been isolated from domestic cats (i.e., *Felis catus*), specifically: *Bartonella henselae* [2–8], *Bartonella clarridgeiae* [9–12], *Bartonella koehlerae* [13], and, on one occasion, *Bartonella bovis* (formerly *Bartonella weissii*) [14]. *B. henselae* is transmitted to humans by scratches or bites from cats or by bites from the cat flea *(Ctenocephalides felis)* [15], and the organism can cause cat-scratch disease (CSD), bacillary angiomatosis, hepatic peliosis, and endocarditis [16]. Other felines, such as mountain lions *(Felis concolor),* bobcats *(Felis rufus),* and Florida panthers, may also be infected by *B. henselae* [17, 18]. *Bartonella* species are responsible for chronic, asymptomatic bacteremia in kittens and adult cats, with a high prevalence of cases—ranging from 4% to 70%—occurring among apparently healthy cats [3, 4, 19]. *B. henselae,* which comprises 3 subspecies or genotypes—the Houston [20], Marseille [21], and Berlin [22] genotypes—that have been identified using multilocus sequence typing [23], is distributed in cats and humans worldwide. Although the Marseille and Houston genotypes have been recovered in humans, genotype Berlin has been identified only in a cat in Germany [24]. Chronic, asymptomatic bacteremia is an exceptional disease among mammals, and *Bartonella* bacteremia in cats is a unique model for the study of the antiquity of the relationship between cats and *Bartonella* species and their coevolution [25]. The detection and analysis of *Bartonella* DNA sequences that were recovered from premodern cat specimens may help to answer these questions. The purpose of the present study was to investigate the presence of *Bartonella* species DNA in the dental pulp of French cats dated from the 13th to 16th centuries.

Methods. We collected a total of 135 teeth from 19 domestic cats from 7 burial sites in France (figure 1). Powdery remnants of dental pulp were scraped off the teeth, and total DNA was then extracted from the dental pulp, as reported elsewhere [26]. All manipulations were performed separately in different laboratories in which only non-*Bartonella* species had been previously manipulated, to avoid any possibility of modern molecular contamination of the premodern DNA samples. Every step in the experiment was performed in a separate room, with the use of disposable equipment and newly prepared reagents. To prevent carry-over of *Bartonella* species, PCR-positive control samples were never used [27], and 3 control teeth were extracted from 3 contemporary cats after verification of the absence of *Bartonella* species DNA by PCR. Also, 3 control assays for contamination, in which the DNA sample was replaced with sterile water, were included for each PCR assay. Amplifications were performed in a 25-μL mixture that was prepared as described elsewhere [28]. Sequences of the primers targeting the *groEL* gene were previously reported elsewhere [29]. Sequences of the primers targeting the *Pap31* gene were PAPF1EXT: 5′-GATTCTAGGAGTTGAAACCGA-3′ (positions 261–281; *Bartonella henselae* [GenBank accession number AF308169]), PAPR1EXT: 5′-ACGCGAGTAGCACCAGACCA-3′ (positions 463–482), PAPF2INT: 5′-TGACAGAGAAGACGCAAAAA-3′ (positions 294–313), and PAPR2INT: 5′-CCTTTAAAGCTAAACTATCTG-3′ (positions 437–457). PCR included denaturation at 94°C for 3 min, followed by 43 cycles of the following sequence: denaturation at 94°C for 30 s, primer

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Reprints or correspondence: Dr. Michel Drancourt, Unité des Rickettsies, CNRS UMR 6020, IFR 48, Faculté de Médecine, Université de la Méditerranée, Marseille, France (Michel.Drancourt@medecine.univ-mrs.fr).

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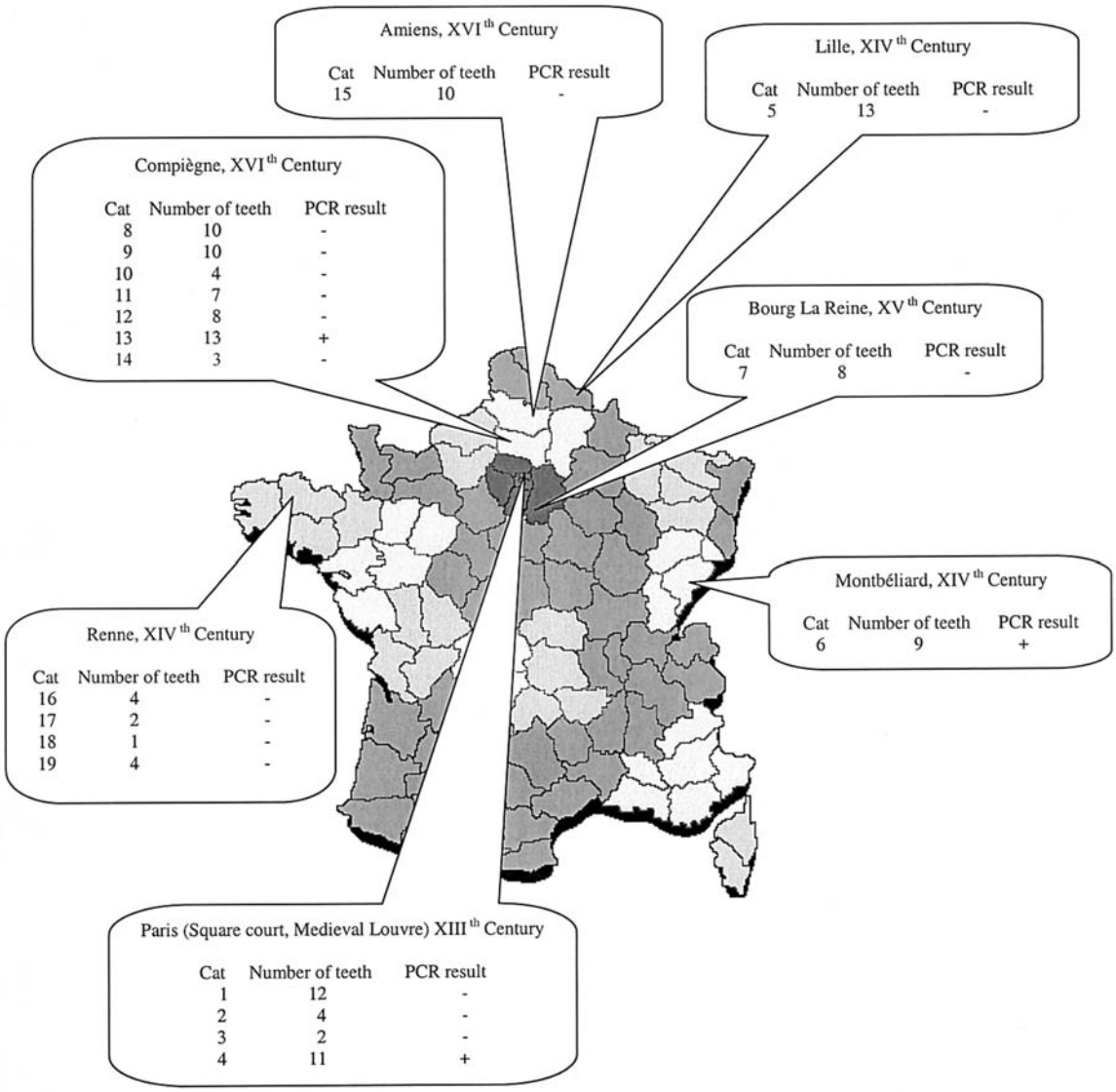

Figure 1. Geographical distribution of premodern French cats used in a study of the antiquity of *Bartonella henselae* infection among domestic cats. −, negative; +, positive.

annealing at 60°C (or at 58°C for HSPps primers) for 30 s, and elongation at 68°C for 90 s. Nested PCR was directly performed in a final volume of 42 μL under the same conditions. The amplification was completed by holding the reaction mixture at 68°C for 7 min to allow complete extension of the PCR products. After purification (Milipore Multiscreen kit; Milipore SAS) and cloning (pGEM-T Easy Vector System II kit; Promega), sequencing reactions were performed using the Big Dye Terminator Cycle Sequencing V2.0 kit (Perkin-Elmer Biosystem) and the 3100 Genetic Analyzer (Applied Biosystem). Sequences were aligned using the multisequence alignment Clustal W, version 1.8, and were compared with sequences that were available in GenBank.

Results. No amplification was obtained for the 3 PCR-negative control teeth or for the 3 contamination controls by use of the PCR assays for both the *pap31* and *groEL* genes. For primers targeting the *groEL* gene, an amplicon of the expected 269-bp size was obtained from DNA extracts that had been derived from the cats that were designated as cats 4, 6, and 13 (figure 1). Sequences derived from clones of cat 4 and cat 13 were identical to the sequence of the modern *B. henselae* genotype Houston *groEL* gene (GenBank accession number AF 304023), whereas sequences obtained from 3 clones of cat 6 were identical to one another and presented 1 mutation in comparison with the *B. henselae* genotype Houston *groEL* gene sequence (A→G) in codon 356, resulting in translation from a lysine to glutamic acid (GenBank accession number AY 5125555) (figure 2). For primers targeting the *Pap31* gene, an amplicon of the expected 164-bp size was obtained from DNA extracts that had been derived from the same 3 cats. The se-

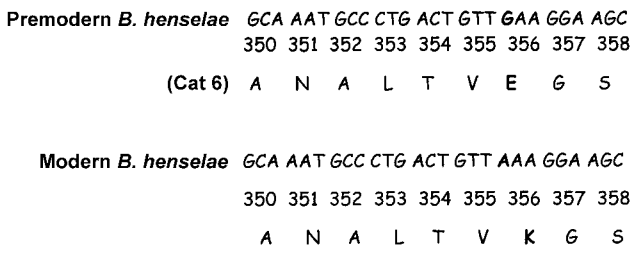

Figure 2. Partial sequence of the *Bartonella henselae groEL* gene, showing a mutation A→G, that was determined in cat 6.

quence of these cloned amplicons were identical to that of the *B. henselae* phage 60457 strain SA-2 *pap* gene (GenBank accession number AF 308168).

Discussion. We detected *B. henselae groEL* and *pap31* gene fragments in the dental pulp of 3 cats dating from the 13th, 14th, and 16th centuries. We believe that the finding of identical sequences did not result from modern contamination of the specimens, because of the extensive precautions that we took. Specimens were manipulated in laboratories in which *Bartonella* species were never manipulated previously, as was recently recommended [30, 31], and no amplification was obtained for the 3 PCR-negative control teeth or for the contamination controls. PCR-positive teeth were tested using 3 different sets of primers that target 2 different regions, and the detection of a unique mutation in the studied *groEL* sequence in 1 of the specimens definitely ruled out contamination by modern *Bartonella* DNA. This mutation was reproduced in 3 clones, which suggests that it did not result from a misincorporation by the *Taq* DNA polymerase but, rather, indicated a true mutation in the premodern sequence.

The present report provides additional evidence of the usefulness of dental pulp for the detection of bloodborne microorganisms. *Coxiella burnetii* DNA and viable *C. burnetii* were recovered from dental pulp of bacteremic guinea pigs that were experimentally infected with *C. burnetii* [32, 33]. In humans, the HIV genome has been detected in the dental pulp of patients with AIDS [34, 35], and we previously found *Yersinia pestis* DNA in the dental pulp of human remains dating from 2 historical plague pandemics [26–28]. The exploitation of dental pulp is a practical tool in the study of bacteremic diseases, because it allows the recovery of DNA from a naturally enclosed cavity without the necessity of decalcification. It should be made clear that dentine is not comparable to dental pulp, because of the absence of a vascularization system in dentine; this explains why dentine offers no advantage over dental pulp. We now extend the use of dental pulp to the detection of premodern pathogenic *Bartonella* species in their reservoir. The detection of premodern pathogens in their reservoir and vector has seldom been employed, apart from the detection of the Lyme disease spirochete *Borrelia burgdorferi* in archived European ticks that had been preserved in ethanol [36, 37] and in archived rodents [38].

B. henselae genotype Houston was found in the 3 PCR-positive cats, which suggests the continuous persistence of this pathogen among French cats for at least 800 years. These data suggest that epidemiological circumstances were present for the occurrence of CSD within a medieval population in France, because archaeological and historical data indicate that the cats that we found to be PCR positive had lived in close contact with people. Enlarged lymph nodes have been extensively reported as scrofula in medieval literature and historical sources [39]. It was believed that the French and English kings had the divine power to cure scrofula by touching persons who appeared to have the disease. Scrofula has been attributed to tuberculosis, but some of the cases may, indeed, have been self-limiting CSD, thus explaining the "cure" achieved by the royal treatment. It is of interest that the formal practice of the ceremonial rite has been traced back to the 13th century in France [39].

Molecular analyses have suggested that *Bartonella* species associated with indigenous rodents of the New World were phylogenetically distinct from *Bartonella* species that have been recovered from Old World rodents, which suggests that species that were separated by continental drift have evolved independently from one another [40]. As for *B. henselae,* 3 genotypes have been reported in Europe; only 2 of these genotypes—the Houston and Marseille genotypes—have been detected worldwide. In the New World, serological evidence indicates that continent-restricted felines, including the bobcat *(F. rufus)* and the mountain lion *(F. concolor),* were infected with an organism that resembles *B. henselae* [17]. Large series of *B. henselae* infections in humans have rarely been reported worldwide. Data show a that a 76.5% majority of *B. henselae* infections in Australia are due to the Houston genotype [41], whereas, in Europe, the Houston and Marseille genotypes were identified at comparable levels in patients with *B. henselae* infection [21, 29]. Current distribution of *B. henselae* genotypes may result from the divergent evolution of the feline-associated *B. henselae* strains that followed continental drift. Additional transcontinental exchanges of genotypes may have occurred after the time of Columbus. Here, we provide evidence that *B. henselae* genotype Houston was present in France before the time of Columbus. This hypothesis warrants further studies, including studies of premodern cat specimens that originated from the New World, as well as from Egypt, where the cat is supposed to have been domesticated first.

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