MOLECULAR DETECTION OF RICKETTSIA FELIS, RICKETTSIA TYPHI AND TWO GENOTYPES CLOSELY RELATED TO BARTONELLA ELIZABETHAE

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Abstract. A total of 56 fleas were collected from mice, rats, and one hedgehog in national parks of mainland Portugal and the Madeira Island. All fleas were tested for the presence of bacteria of the genera Rickettsia and Bartonella using PCR assays. In fleas from mainland Portugal, we detected Rickettsia felis in one Archaeoppysla erinacei maura flea and in one Ctenothalmus sp. In five Leptopsylla segnis fleas taken from rats in the Madeira Island, we identified Rickettsia typhi. In addition, in four fleas from the genera Ornithophaga and Stenoponina collect from mice and a rat in mainland Portugal, we detected the presence of two new Bartonella genotypes closely related to Bartonella elizabethae. Our findings emphasize the potential risk of flea-transmitted infections in mainland Portugal and the Madeira archipelago, and extend our knowledge of the potential flea vectors of human pathogens.

INTRODUCTION

In suburban and urban areas worldwide, fleas are most often associated with human dwellings through their natural hosts, such as rats and mice, and peridomestic animals. Fleas are vectors of several pathogens including Yersinia pestis, Rickettsia typhi, Rickettsia felis, and Bartonella henselae,1,2 which are the etiologic agents of plague, murine typhus, flea-borne spotted fever, and cat scratch disease, respectively.3,4 Murine typhus is widely distributed worldwide and mainly occurs in coastal areas, especially in harbors. In Portugal, murine typhus was first recognized during the 1940s.5,6 After this period, the disease was not reported until 1994 when 2 clinical cases were reported in the Lisbon area.7 Then, in November 1996, a murine typhus outbreak was investigated in the Madeira Archipelago. Twelve patients were serologically confirmed as having R. typhi infection by the Portuguese National Institutes of Health.5,7 The classic cycle of murine typhus involves rats, Rattus rattus and Rattus norvegicus, as reservoirs, and their fleas, with the oriental rat flea, Xenopsylla cheopis, being the main vector.10 R. typhi is transmitted to humans by contamination of the bite site or contact with flea feces aerosols. Other reservoirs such as the opossum and vectors such as the cat flea Ctenocephalides felis have also been described and may be involved in the epidemiologic cycle of murine typhus.11 R. felis, formerly named ELB agent, was detected in 1990 for the first time in C. felis,12 and has recently been recognized as the agent of flea-borne spotted fever.13 To date, R. felis has been found in several countries including the United States,13 Mexico,14 Brazil,15 Peru,16 New Zealand,17 Thailand,18 Germany,18 France,13 United Kingdom,19 and Algeria.20

Bartonella species have been identified as important zoonotic agents causing various human diseases, and are associated with multiple arthropod vectors including fleas, which are able to carry several Bartonella species.4,21–24

In the present study, we report the molecular detection of R. typhi, R. felis and 2 new Bartonella genotypes related to B. elizabethae in fleas collected from rodents and 1 hedgehog in mainland Portugal and the Madeira Archipelago.

MATERIALS AND METHODS

Study sites and sample collection. The fleas tested in this study were collected on 2 separate expeditions. The first took place in Porto Santo (33° 04'0"N, 16° 20'28"W), Madeira Archipelago Island in January of 1997 during a murine typhus rodent serosurvey. In the second study, fleas were collected from Mafra (38° 56'N, 09° 17'W), Bucelas (38° 54'02"N, 09° 07'08"W), Arrábida (38° 28'23"N, 09° 01'58"W), and Mêrtola (37° 32'N, 07° 48'W), all in mainland Portugal, during a research project to study lymphocytic choriomeningitis virus and hantavirus in rodents. During this period, rodents were live-trapped and combed for the presence of fleas, and the fleas were kept frozen at −80°C in sterile tubes without any additive until being further processed.

Flea sampling. A total of 56 fleas were collected from 20 rodents and 1 hedgehog. Thirty-eight fleas were collected from 10 Rattus rattus and 1 Rattus norvegicus from Porto Santo Island, Madeira Archipelago. In addition, 18 fleas from 2 rats (1 R. rattus and 1 R. norvegicus), 7 mice (1 Mus musculus, 5 Mus spretus, and 1 Apodemus sylvaticus), and from the hedgehog (Erinaceus europaeus) were collected in mainland Portugal (Table 1).

Flea identification. Fleas were identified on the basis of morphometric characteristics by one of the authors (MS).

DNA extraction. Fleas were washed for 5 minutes in iodinated alcohol and then in sterile distilled water for 5 minutes before being dried on sterile filter paper. DNA was extracted using alkaline hydrolysis as described previously for ticks.25 As negative control we included during DNA extraction from flea samples sterile water specimens using a ratio of 1 control for every 6 samples. DNA extracts were stored at −20°C until further processing.

Polymerase chain reaction (PCR). DNA from each flea was tested by PCR using the primers Rp877F and Rp1258R, which amplify a 381-bp fragment of the citrate synthase-encoding gene (gltA) from Rickettsia sp.26 and the primers QHVE1 and QHVE3, which amplify a fragment of variable size of the 16S-23S rRNA intergenic spacer (ITS), depending on the Bartonella species.27 Negative controls were further
processed by PCR as tick specimens. To avoid contamination, no positive controls were used. Positive PCR results were confirmed using PCR assays incorporating the 120-M59 (5'-H11032-CCGCAGGRTTGTAAC7GC-3') and 120-807 (5'-H11032-CCTTTTAGATTACRCCTAA-3') primer pair, which amplify a 833-bp fragment of the rOmpB protein-encoding gene (ompB) from Rickettsia species, and the BhCS.781p and BhCS.1137n primers, which amplify a 339-bp fragment from the gltA gene from Bartonella species, respectively. PCR amplifications were carried out in a PTC-200 Peltier Thermal Cycler (MJ Research, Inc., Watertown, MA). Each PCR mixture consisted of the following: 5 µl of DNA, 12.5 pmol of each primer, 200 µM of dATP, dCTP, dTTP, dGTP, 1U of Elongase in 1X PCR buffer with 0.8 µl of 25 mM MgCl2 (Life Technologies, Cergy Pontoise, France), and sterile distilled water to a final volume of 25 µL. PCR cycles included an initial 3-minute denaturation step at 94°C, followed by 44 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, and extension at 68°C for 90 seconds. Amplification was completed by holding the reaction mixture at 68°C for 7 minutes to allow complete extension of PCR products. PCR products were resolved by electrophoresis in 1% agarose gels, and when appropriately sized products were found, they were purified by using the QIAquick Spin PCR purification kit (QIAGEN) prior to sequencing.

Sequencing and phylogenetic analysis. Positive PCR products were sequenced using PCR primers and the d-Rhodamine Terminator Ready Sequencing kit and an ABI 3100 automated DNA sequencer ( Applied Biosystems) according to the manufacturer’s instructions. Sequences were identified using the BLAST feature of GenBank. Phylogenetic relationships among sequences from Portuguese fleas, representatives of the established Bartonella taxa, and other partially characterized Bartonella isolates were analyzed using the neighbor-joining method with Kimura-2 parameter within the Mega 2.1 software.

RESULTS

Flea identification. The 38 fleas collected in Porto Santo Island were morphologically identified as 14 Xenopsylla cheopis, 21 Leptopsylla segnis, 2 Ctenocephalides spp., and 1 Nosopsyllus fasciatus. Among the 18 fleas collected in mainland Portugal, 8 fleas were morphologically identified as Stenoponia tripectinata, 7 as Typhloceras poppei poppei, 1 as Ctenophilalus spp., 1 as Ornithophaga spp., and 1 as Archaeopsylla erinacei maura (Table 1). Figure 1 shows the areas in mainland Portugal where rodents and fleas were collected.

**Polymerase chain reaction and sequencing.** Overall, PCR amplicons were obtained from 11 (19.6%) of the 56 tested fleas. Both the gltA and ompB-based PCR assays produced amplicons of the expected sizes in 7 (12.5%) fleas. A rickettsia exhibiting gltA and ompB sequences 100% similar to those of R. felis was identified in the Ctenophilalus sp. and A. erinacei maura fleas collected in mainland Portugal (Table 1). A rickettsia with 100% sequence similarity to the gltA and ompB genes of R. typhi was identified in 5 of the 21 (23.8%) L. segnis fleas collected on Porto Santo Island.
Both the ITS and gltA-based PCR assays detected Bartonella spp. in 4 (7.1%) of all tested fleas. These 4 fleas (i.e., 3 S. tripectinata and 1 Ornithophaga sp.) had been collected in mainland Portugal. All 4 fleas had ITS amplicon sizes of 576 bp. Amplicons from the 3 S. tripectinata fleas exhibited identical sequences and exhibited 93.0% (536/576 bp) and 97.9% (331/339) nucleotide pairwise similarities with the ITS and gltA sequences, respectively, of B. elizabethae, their closest validated Bartonella species. The amplicon from the Ornithophaga sp. flea exhibited 92.4% (532/576) and 97.0% (329/339) ITS and gltA sequence pairwise similarities, respectively, with B. elizabethae. The 2 new Bartonella genotypes detected in S. tripectinata and Ornithophaga sp. differed by 4 (99.3% similarity) and 2 (99.4%) nucleotide mutations for the ITS and gltA sequences, respectively. Sequences were deposited in GenBank under the accession numbers AY877424 and AY877422 for the ITS and gltA fragments amplified from S. tripectinata fleas, respectively, and AY877425 and AY877423 for the ITS and gltA fragments amplified from the Ornithophaga spp. flea, respectively. The phylogenetic analysis of Bartonella amplicons demonstrated that the 4 flea amplicons clustered with B. elizabethae with a significant bootstrap value (Figure 2).

**Figure 2.** Unrooted dendrogram showing the phylogenetic position of Bartonella sp. detected in Stenoponia tripectinata and Ornithophaga sp. fleas collected in Portugal. The dendrogram was obtained using the neighbor-joining parsimony method with Kimura 2 parameter within the Mega 2.1 software, after alignment of a portion of the gltA gene of the flea amplicons obtained in this study and sequences present in GenBank. GenBank accession numbers are indicated in parentheses. Bootstrap values above 75% are indicated at relevant nodes. The scale bar represents a 2% nucleotide sequence divergence.
DISCUSSION

We report the detection of 3 flea-associated bacteria in Portugal, 2 of which are recognized as human pathogens, based on the concordant results of 2 different PCR amplifications for each of the investigated bacterial genera.

To date, murine typhus is the only flea-borne rickettsiosis that has been identified in Portugal. Although murine typhus is a reportable disease, its real incidence is not clear, particularly in the Madeira Archipelago. The most recent cases were reported during an outbreak in November and December of 1996 in Porto Santo Island in which 5 cases were diagnosed serologically. After this outbreak, in January 1997, the Portuguese National Institutes of Health performed a serological survey in the population of Porto Santo, and found a prevalence of 27% to antibodies to R. typhi (IgG and/or IgM) among tested serum samples. Concomitantly, a seroprevalence of antibodies to R. typhi of 76% was found in rodents captured in the same area.

At that time, rodent ectoparasites were collected but were not tested for the presence of R. typhi. After 6 years we demonstrated that R. typhi was present in rat fleas collected in 1997 in that area. Since then, the island Regional Health Directorate performed a systematic control of the number of rats and their ectoparasites and, to date, no more murine typhus cases have been reported. Nevertheless, we cannot exclude the possibility that undiagnosed cases occurred during this period.

R. typhi was only detected in L. segnis fleas and although this flea species has not been considered an important vector of infectious diseases because it does not bite humans, laboratory studies have shown that L. segnis is more effective in transmitting R. typhi to rats than its most common vector, X. cheopis. Our study has confirmed a prevalence of 23% in infected L. segnis fleas. Therefore, it is suggested that L. segnis may play a role in the epidemiologic cycle of murine typhus in Madeira. In addition, L. segnis is able to remain attached to the skin of the rat for several hours or days and may have the opportunity to acquire heavier rickettsial doses from infected hosts when compared with fast feeder fleas such as X. cheopis. As a consequence, the accumulation of infected flea feces in the fur of infected rodents may increase the risk of human and rat transmission of R. typhi through infected aerosols. Another argument that favors the potential role of L. segnis as a vector of R. typhi is the fact that the murine typhus outbreak in Porto Santo Island occurred in winter, when the activity of L. segnis fleas is the most intense. In contrast, X. cheopis fleas are mostly active in the late spring or early fall.

This report is the first to identify R. felis in fleas collected in Portugal and also the first report of this rickettsia in Ctenophtalmus sp. We also described R. felis in A. erinacei maura fleas corroborating the data published by Bitam and colleagues. The first described and main vector of R. felis is the cat flea C. felis. Nevertheless, this bacterium has also been found in C. canis and Pulex irritans, and our data highlight the existence of other flea species that are able to harbor this rickettsia, thus corroborating data from other authors about the possibility of R. felis infecting other rodent-associated flea species. An interesting finding is that all of the single fleas A. erinacei maura and Ctenophtalmus spp. collected from different regions and animals were positive for R. felis. This fact suggests a high infection rate in different regions. During this study the hedgehog was accidentally trapped in one of the rodent cages and the flea collected from this animal was positive for R. felis. This may indicate that animals other than wild rodents may be able to serve as reservoirs for R. felis. Furthermore, a recent study showed that Chlamydia sp., Coxiella burnetii, Yersia spp., and other bacteria might be transmitted by hedgehogs and probably by its ectoparasites. Nevertheless, further studies must be performed in the same area to collect more fleas to better evaluate the prevalence of infection.

We found that 37% of Stenoponia tripecinata and the single Ornithopahaga spp. fleas collected in R.rattus and M. spretrus mice (Algerian mice) were infected with Bartonella genotypes related to the recognized human pathogen B. elizabethae (Fig. 2). B. elizabethae was isolated for the first time in 1986 from the blood of a patient with severe endocarditis in Massachusetts and in 1996, a serosurvey identified antibodies to B. elizabethae in intravenous drug users from Maryland. Subsequently, B. elizabethae or closely related isolates were detected in rats and other rodent species in Peru and the United States, China, Bangladesh, Thailand, and South Africa. Therefore, although rodents are clearly identified as the hosts of B. elizabethae, prior to our study, no insect vector had been identified for this species and our results demonstrate that 2 rodent flea species are potential vectors of B. elizabethae and might play roles in human infection.

Through the identification of 3 human pathogens in rodent fleas in Portugal, including 2 described for the first time in the country, we emphasize the necessity of including R. felis and Bartonella spp. in the differential diagnosis of flea-borne infections in Portugal, in addition to R. typhi. These results have expanded our knowledge about flea vectors and will provide us with useful data about non-traditional vectors of rickettsial diseases and also the possible pathogens they may transmit to humans in Portugal.

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REFERENCES


