FIRST MOLECULAR DETECTION OF RICKETTSIA FELIS IN FLEAS FROM ALGERIA

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Abstract. Fleas collected in Algeria in the district of Oran between July and September 2003 were tested by polymerase chain reaction for the presence of Rickettsia spp. DNA using primers amplifying gltA and OmpA genes. Two gltA sequences identical to those of an emerging pathogen, Rickettsia felis, were detected including i) R. felis California 2 in Ctenocephalides canis from rodents and ii) R. felis RF2125 in Archeopsylla erinacei from hedgehogs.

INTRODUCTION

With the exception of the rat flea Xenopsylla cheopis and Yersinia pestis, the agent of plague, fleas and human flea-borne infections have been scarcely studied in Algeria.1 Murine typhus, a typhus group rickettsiosis induced by Rickettsia typhi and transmitted by X. cheopis, is also known to occur in Algeria but cases are poorly documented.2,3 However, in recent years, fleas have been associated worldwide with emerging human infections including flea-borne spotted fever (also called cat flea typhus) caused by a spotted fever rickettsia, Rickettsia felis,4 and cat scratch disease in connection with Bartonella henselae.5 In June 2003, the Ministry of Health, Algeria, reported an outbreak of plague in the Oran district.6 Investigations including an entomological survey were conducted to identify and describe a possible natural focus as well as the mechanisms of spread of Y. pestis in this area. During the same period, clinicians working in the Infectious Diseases Unit at the Oran Hospital claimed of also having admitted several patients with spotted fever in the past month, leading to suspect a potential Rickettsia infection. The etiological agents responsible for the disease were unknown, but they may be associated with enzootic vectors, such as fleas or ticks. The aim of the current work was to subsequently survey fleas collected in the District of Oran and detect rickettsial DNA by means of polymerase chain reaction (PCR) involving gltA and OmpA rickettsial genes.

MATERIALS AND METHODS

Sampling area and hosts. The sites that represented the original focus of the reported plague cases were Kehailia and Tafaraoui, both located in the District of Oran, ca. 450 km west of the capital Algiers. This settlement area is situated at an altitude of approximately 208 m above sea level and has around 1,400 inhabitants. The predominant occupation is agriculture, and a large number of domestic and peridomestic animals (dogs, goats) populate the area. Fleas were collected on rodents and hedgehogs trapped inside the houses and in the peridomestic areas of the cities of Kehailia and Tafaraoui. The sampling comprised 57 specimen collected on 3 hedgehogs (Atelerix algirus) in Tafaraoui and 86 specimens collected in Kehailia on rodents including 11 Meriones shawii, 8 Rattus rattus, and 15 Rattus norvegicus.

Collection and identification of fleas. Most of the fleas were used for a survey on plague. However, a total of 4 fleas collected on hedgehogs and 11 fleas collected on rodents were randomly chosen, stored in 70% alcohol, and sent to the WHO Collaborative Center for Rickettsial Reference and Research in Marseille, France, where the molecular studies were performed in February 2004. Alcohol-preserved fleas were rinsed with distilled water for 10 minutes and dried on sterile filter paper in a laminar flow hood. Preliminary entomological identification was done by an entomologist (I.B.), using reference taxonomic keys.7 Voucher specimen could not be retained, as whole specimen extractions were performed.

Polymerase chain reaction and sequencing. Fleas were crushed individually in sterile Eppendorf tubes with the tip of a sterile pipette. DNA was extracted by using the QIAamp Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Rickettsial DNA was detected by PCR using primers RpCS.877p (5'-GGGGACCTGCT-CACCGGCCG-3') and RpCS.1258n (5'-ATTGCAAAAAG-TACAGTGAACA-3') (Eurogentec, Seraing, Belgium), which amplify a 396-bp fragment of the citrate synthase gene (gltA) of Rickettsia as previously described.8,9 Additionally, a 629–632 base-pair fragment of the ompA gene was amplified using Rr190.70p (5'-ATGGGAATATTTTCTCACAAG-3') and Rr190.70n (5'-GGGGACCTGCT-CACCGGCCG-3'). Negative controls consisted of DNA extracted (in the same biosafety cabinet) of live uninfected lice from colonies of our laboratories (1 louse for 5 tested Siphonaptera). A positive control (Rickettsia montanensis) was included in each test. To identify the detected Rickettsia sp., PCR products were purified and DNA sequencing was performed as previously described.8,9 Additionally, we sequenced species-level information of siphonapteran 18S rDNA to ensure correct species diagnosis on a molecular level. Sequences were generated using 18S a0.7 (fw) and 18S bi, b0.5, and b3.0 (all rev) primers, as outlined in Whiting.10 Obtained sequences were edited and because alignment was trivial, manually aligned using Sequencer 4.2 (GeneCodes 2003). The data were compared in phylogenetic (rodent fleas) and statistical frameworks (Maximum parsimony [MP], and pairwise base differences; PAUP*) to flea sequences available in the 18S rDNA database of the Whiting laboratory (Ctenocephalides canis 2; Ctenocephalides felis felis; not yet published) and

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Genbank (Ctenocephalides canis [AF423914]; Ophthalmpsylla volgensis [AF423895]; Pulex irritans [AF423915]; Ctenophthalmus pseudoagyrites [AF423892]; and Panorpa communis [AF423900, outgroup]).

Nonparametric bootstrap values (10,000 replicates, 100 random additions per replicate) were calculated for data sets to assess confidence in the resulting relationships. Independently, all obtained sequences were compared with those available in GenBank by using the nucleotide-nucleotide BLAST (blastn) program (see http://www.ncbi.nlm.nih.gov/BLAST/).

RESULTS

Using morphologic taxonomic keys, the four fleas that had been collected on hedgehogs Atelerix algirus were identified as Archaeopsylla erinacei. This was unambiguously confirmed when a 331 fragment of the 18S RNA showed 100% sequence similarity with a previously deposited sequence of this flea in GenBank (X89486). The morphologic identification of the fleas collected on rodents however remained doubtful. A 918 base pair 18S RNA was shown to have 99.89% similarity (but one gap) with the sequence of Ctenocephalides canis deposited in GenBank (AF423914). Comparisons of pairwise base differences with 177 entries in the database of one of us (K.D.D.L.C.) covering all known flea families showed a 0.4% sequence difference to the closely related C. felis felis, and between 0.5% and 4.8% difference to the rest of the taxa. Additionally, MP analysis under 1,000 replicates of heuristic search with random addition sequence and tree bisection and reconnection branch swapping (TBR) revealed the grouping of the sampled sequence, the previously deposited GenBank sequence, and the yet unpublished sequence from the database (C. canis 2) as a monophyletic polytomy. C. felis felis forms a sister group to all C. canis (Figure 1). All relationships are recovered with high nodal support values. Therefore, fleas collected on the Algerian rodents were ultimately identified as C. canis.

Using the glt A primers, PCR products of rickettsial DNA were detected in all of the 15 studied fleas and from the positive controls. No nucleic acids were amplified from the negative controls. All fleas were also shown to be positive with the second PCR screening using OmpA primers. By sequencing the gltA amplified fragments, two sequences of the expected size were identified. They were shown to be respectively 100% identical to two sequences deposited in GenBank originating from R. felis including i) R. felis California 212 (AF210692) for those detected in the 11 C. canis fleas and ii) Rickettsia RF2125, considered to be R. felis9 (AF516333), from those detected in the 4 A. erinacei fleas.

DISCUSSION

We present here the first molecular detection of R. felis in Algeria. By using primers targeting two different rickettsial genes and appropriate positive and negative controls, measurements were taken to detect genuine Rickettsia infection of the fleas under study. No nucleic acids were amplified from the negative controls. Furthermore, two different sequences were identified from the PCR products that were amplified during the same experiment, thus significantly reducing the possibility of potential contamination with DNA products. Additionally, the ethanol in which the fleas were stored in Algeria originated from a new bottle and had never been used before. However, fleas of the same species collected for this work were stored in the same vial. Also, only a small number of specimens were tested in this work. Thus, we cannot speculate about the high infestation rate of R. felis infection of the fleas tested here.

R. felis is an obligate intracellular Gram-negative bacteria belonging to the spotted fever group of genus Rickettsia within the order Rickettsiales.13 It is known as the agent of flea-borne spotted fever, an emerging disease, which is to date only incompletely described.4 R. felis was probably first detected in cat fleas Ctenocephalides felis in 191814 and rediscovered in 1990.15 This bacterium was characterized by molecular biology techniques and was initially named the ELB agent (EL Laboratory, Soquel, CA). In 1994, DNA fragments of this agent were detected in blood samples obtained from a patient from Texas in 1991.16 The name R. felis was proposed after the first attempts to cultivate and characterize the in vitro and in vivo antibiotic susceptibilities of the ELB agent were reported in 1995 and 1996.17,18 Nevertheless, the first definitive cultivation of this low temperature growing R. felis was only achieved in 2001.12,19,20

More arguments on the pathogenicity of R. felis for humans were provided in 2000 in Mexico, when three patients with fever rash were diagnosed with R. felis infection by specific PCR of blood or skin and a seroconversion to rickettsial antigens.21 Serological evidence of R. felis infection has also been shown in patients from France and Brazil.12 Moreover, molecular documentation was obtained in the serum of one Brazilian patient.12 In 2002, two cases of typical rickettsial spotted fever including generalized maculopapular rash and a black eschar were reported in an adult couple in Germany.22 R. felis infections were documented by serology for both patients and by detection of R. felis DNA in the woman’s sera.22 Finally, the first case of R. felis infection was recently documented by serology in Asia.23 Rash and/or eschar (6 of 8) have been reported in the few cases documented. Interestingly, these clinical features are known in the Mediterranean area including Algeria as typical signs of the Mediterranean spotted fever (MSF), a tick-borne spotted fever rickettsiosis.
due to *R. conorii* and transmitted by the brown dog tick, *Rhipicephalus sanguineus*.\(^\text{24}\)

In recent years, *R. felis* has been associated with fleas throughout the world. It has been detected in fleas in Brazil\(^\text{25}\), Ethiopia\(^\text{3}\); in Europe including Spain,\(^\text{30}\) France,\(^\text{8}\) and the United Kingdom\(^\text{27,28}\), in Thailand,\(^\text{9}\) and in New Zealand.\(^\text{29}\) To date, three species of fleas have been associated with *R. felis*, namely *C. felis*,\(^\text{8,9,25-26}\) *C. canis*,\(^\text{9}\) and *Pulex irritans*.\(^\text{30}\) Interestingly, transversal transmission of *R. felis* in fleas has also been reported, suggesting that fleas could act as reservoirs of the rickettsia.\(^\text{30}\) As taxonomical identification of fleas to the species level is difficult and can only be undertaken by experts, which are ever more scarce, and given the fact of a new flea association for *R. felis*, we used 18S rRNA sequence analysis to confirm identification of the fleas. Although rodents are not a common host for *C. canis*, many dogs (the usual hosts) were around the places where the rodents were trapped. To our knowledge, this is the first time that *A. erinacei* is associated with *R. felis*, but some collaborators in Portugal have also recently detected *R. felis* in this species of fleas collected on hedgehogs in Portugal. (R. Sousa and others, unpublished data). Although these data suggest that *R. felis* infection may be prevalent worldwide, the role of mammals, including rodents, hedgehogs, cats, and dogs, in the life cycle and circulation of *R. felis* is still unclear.

Our results suggest that *R. felis* infection is prevalent in Algeria and that fleas are potential vectors of human rickettsioses in this country. This is the second time that this emerging pathogen was detected in Africa. Further studies are needed to elucidate and describe the epidemiology of *R. felis* infection. Clinicians, however, in Algeria or those elsewhere who may see patients returning from this country, must now be aware of possible *R. felis* infection in patients presenting signs of spotted fever and/or an eschar. Although it may be misdiagnosed as and treated similar to the tick-borne Mediterranean spotted fever, the epidemiologic aspects of flea-transmitted diseases regarding the risk of exposure as well as prevention aspects are different and need consideration. To date, few confirmed cases of *R. felis* infections have been described throughout the world, including none in Algeria. A better description of this emerging disease may allow recognizing subtle clinical differences to MSF.

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