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Original article

Bacterial agents in 248 ticks removed from people from 2002 to 2013

Camille Aubry, Cristina Socolovschi, Didier Raoult, Philippe Parola*

Aix Marseille Université, Unité de Recherche en Maladies Infectieuses et Tropicales Emergentes (URMITE), UM63, CNRS 7278, IRD 198 (Dakar), Inserm 1095, Marseille, France

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ABSTRACT

A retrospective study was conducted to analyze the tick species removed from people and to detect tick-infecting bacteria in the specimens collected over the past 10 years at the reference center for rickettsioses, Marseille, France. A total of 248 ticks were removed from 200 people, including *Dermacentor* (73), *Rhipicephalus* (67), *Ixodes* (60), *Amblyomma* (8), *Argas* (3), *Hyalomma* (1), and *Haemaphysalis* (1) species. Bacterial DNA was detected in 101 ticks: *Rickettsia slovaca* (34%) and *Rickettsia raoultii* (23%) were detected in *Dermacentor* ticks; *Rickettsia conorii* (16%) and *Rickettsia massiliae* (18%) were found in *Rhipicephalus* ticks; and *Anaplasma phagocytophylum* (5%), *Borrelia* spp. (8%) and *Rickettsia* spp. (2%) were detected in *Ixodes* ticks. Among the bitten people for which clinical data and laboratory samples were available, tick borne diseases were confirmed in 11 symptomatic individuals.

1. Introduction

Ticks are obligate hematophagous arthropods that parasitize four of seven classes of Vertebrata in almost every region of the world and occasionally bite humans (Parola and Raoult, 2001). Ixodid ticks, also called hard ticks, are the main vectors of human infectious diseases in European countries. They transmit a number of different pathogens, including bacteria, viruses, and protozoa, which cause disease in humans (Jongejan and Uilenberg, 2004; Parola and Raoult, 2001). *Borrelia burgdorferi* sensu lato, the agent of Lyme disease (Stanek et al., 2012), and *Rickettsia conorii conorii*, the agent of Mediterranean spotted fever (MSF), were considered the main tick-borne bacterial pathogens in Europe until recently, when new pathogens transmitted by ticks were described (Parola et al., 2013). For example, the most common tick-borne rickettsioses in Europe are currently caused by *Rickettsia slovaca* and *Rickettsia raoultii*, which cause SENLAT syndrome (scalp-eschar and neck lymphadenopathy after tick bite). These rickettsiae, which are transmitted by *Dermacentor* ticks, were described as human pathogens in 1997 and 2006, respectively (Parola et al., 2013).

The ability of ticks to attach to humans and transmit pathogens is influenced by several factors, including tick abundance, geographical and climatic conditions, human activities, tick burden, biological stages, and duration of tick attachment (Parola and Raoult, 2001). The removal of a tick from a person by that person or a physician is clinical event encountered in the practice of every general practitioner, infectious disease specialist and dermatologist.

The National Reference Center for Rickettsial Diseases in Marseille, France, receives human samples and arthropod specimens mostly from France but also from other parts of the world for tick-borne disease diagnosis. Ticks removed from people, including symptomatic patients, are identified and then screened for the presence of bacteria with molecular tools to give advice to patients, clinicians and microbiologists about the risk of tick-borne diseases and the necessary precautions to take. Here, we present the results of molecular analyses of tick-infecting bacteria and the identification of ticks removed from people and received by our institution over the last decade.

2. Materials and methods

2.1. Tick identification

All ticks removed from humans and analyzed in our laboratory between February 13, 2002 and May 13, 2013 were included in this study. They were morphologically identified using standard taxonomic keys (Estrada-Peña et al., 2005; Hillyard, 1996; Walker et al., 2003). From 2005, when morphological identification was not possible (due to a damaged tick, absence of an entomologist, etc.) or when a tick was found to harbor bacteria, tick identification has been done by standard PCR assays targeting the 12S rRNA gene

* Corresponding author at: Unité de Recherche en Maladies Infectieuses et Tropicales Emergentes (URMITE), Faculté de Médicine, Boulevard Jean Moulin, 13005 Marseille, France.
E-mail address: philippe.parola@univ-amu.fr (P. Parola).

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(360-bp fragment) (Socolovschi et al., 2012b). Additionally, from March 2012, protein profiling by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) was introduced as an additional tool for the rapid identification of tick vectors (Yssouf et al., 2013).

2.2. Molecular detection of bacteria in ticks

DNA samples were extracted from half of each tick with QIAamp Tissue Kits (QIAGEN, Hilden, Germany), following the manufacturer's instructions. The genomic DNA was stored at −20 °C until its use as a template in PCR assays. The unused tick body parts were stored at −80 °C for subsequent analyses. Master mixes were prepared with a QuantiTect Probe PCR kit (QIAGEN) following the manufacturer's instructions. DNA samples extracted from uninfected ticks from a laboratory colony were used as negative controls (Sarih et al., 2008).

Before 2007, tick samples were screened for the presence of rickettsial DNA by conventional PCR targeting a 750-bp fragment of the *gltA* gene, and the positive samples were confirmed by amplifying and sequencing a 632-bp fragment of the *ompA* gene as previously described (Sarih et al., 2008). After that time, quantitative real-time PCR (qPCR) targeting a fragment of the *gltA* gene (Socolovschi et al., 2012b) was used for screening all the *Rickettsia* species of the spotted fever group (SFG), with subsequent specific qPCR reactions depending on the species of the ticks testing positive. The *Rickettsia* positive *Rhipicephalus* ticks were subsequently tested by qPCR specific to *Rickettsia massiliae* and *R. conorii* (Mouffok et al., 2011); positive *Dermacentor* ticks were tested by qPCR specific to *R. slovaca* and *R. raoultii* (Bechah et al., 2011). For other *Rickettsia* positive ticks where it was not possible to determine the bacterial species by specific qPCR, amplification and sequencing of the *gltA* and *ompA* genes were performed as described previously (Sarih et al., 2008). In addition, all DNA samples were screened by qPCR for *Bartonella* spp. by targeting an internally transcribed spacer (Angelakis et al., 2010), for *Coxiella burnetii* using IS30A spacers (Mediannikov et al., 2010), for *Borrelia* spp. by targeting a fragment of the *16S* rRNA gene (Parola et al., 2011), and for *Francisella tularensis* by targeting the *yqaB* gene (Angelakis et al., 2009). *Bartonella* positive ticks were subsequently tested by *B. henselae*-specific qPCR (Angelakis et al., 2010). *Borrelia* positive ticks were confirmed positive by conventional PCR with primers Bor1-Bor2 which enabled amplification of the flaB gene, as described and the sequence of the amplified fragments were obtained (Assous et al., 2006).

The DNA of bacteria within the Anaplasmataceae family was detected by conventional PCR using the primer set EHR16SR–EHR16SD, which amplifies a 345-bp fragment of the 16S rRNAgene (Parola et al., 2000). "*Coxiella*-like" bacteria were identified by qPCR using the primers CL-Rhf, 5′-ACC-TAC-CCT-TGA-CAT-CCT-CGG-AA-3′ and CL-Rhr, 5′-GCA-ACT-AAG-GAC-GAG-GGT-TG-3′, and the CoxL probe, 6-FAM-CAG-CTC-GTG-TCG-TGA-GAT-GT-TAMRA.

Rickettsia montanensis DNA served as a positive control for the primer and probe set targeting SFG Rickettsia. For other bacteria, DNA extracted from the cell-culture supernatant of each agent species served as a positive control for the corresponding primer and probe set. DNA extracted from *Coxiella*-like positive ticks from the laboratory colony was used as a positive control.

All the amplified products of conventional PCR assays were sequenced as described above (Socolovschi et al., 2012b). Sequences were analyzed using ChromasPro, version 1.3 (Technelysium Pty, Ltd., Tewantin, Queensland, Australia) and were compared with sequences from GenBank.

2.3. Bitten people and patients

For each tick received, clinicians were contacted by phone to obtain clinical and epidemiological data about the individuals who had been bitten. This information included age, sex, place of exposure, contact with animals, the presence of fever, rash, an inoculation eschar and/or lymphadenopathy and treatment. For symptomatic patients, acute and convalescent sera (100 μl) were requested for serology assays; EDTA blood samples (200 μl), and skin biopsies (2–4 mm in diameter) or eschar crusts/swabs (200 μL of culture medium solution) were requested for PCR amplification. The analyzed samples were collected at the time of the tick bite discovery, or early clinical signs, or 2–3 weeks after recovery when possible. All sera were screened by immunofluorescence assays (IF) using antigens from the spotted fever group (SFG) species *Rickettsiae*, *Bartonella quintana*, *B. henselae*, *Anaplasma phagocytophilum*, *B. burgdorferi*, *F. tularensis* and *C. burnetii* phase I and II, as previously described (Dubourg et al., 2014; Mediannikov et al., 2010; Fournier et al., 2005). For *Rickettsiae*, *F. tularensis*, *Bartonella* spp. and *A. phagocytophilum* serology, titers of 1:64 for IgG and 1:32 for IgM were used as cutoff values but for acute Q fever, a titer of anti-phase II IgG antibodies of 1:200 and a titer of anti-phase II IgM antibodies of 1:50 was used (Mediannikov et al., 2010). Western blotting procedures were performed as described elsewhere (Dubourg et al., 2014; Fournier et al., 2005). DNA extracted from human samples was tested for all the bacterial pathogens as described above for tick specimens. A database was established in Microsoft Excel and subsequently analyzed.

3. Results

3.1. Tick identification

A total of 248 ticks (90% collected from people bitten in France) from a population of 200 individuals were analyzed in this study. Of these, 27% were sent by a general practitioner, 1% by the individual, 33% by infectious disease consultants, 9% by biologists, 5% by dermatologists, and 7% by emergency room consultants. The medical specialists who sent the remaining 18% of ticks could not be identified. Most of the specimens (213/248) were identified as *Dermacentor*, *Rhipicephalus* and *Ixodes* ticks (34% (73/213), 32% (67/213), and 28% (60/213), respectively) (Table 1). Unfortunately, morphological identification was not found in the laboratory archive for 35 specimens of the 248 received ticks (14%). Tick specimens identified to the species level included 63 *Dermacentor marginatus*, 61 *Rhipicephalus sanguineus*, 2 *Rhipicephalus bursa*, 38 *Ixodes ricinus*, 2 *Ixodes acuminatus*, 2 *Ixodes hexagonus*, 3 *Amblyomma variegatum*, 1 *Amblyomma americanum*, 1 *Amblyomma cajennense*, 1 *Amblyomma hebraeum*, 1 *Amblyomma tapirellum*, 2 *Argas reflexus*, and 1 *Hyalomma lusitanicum* (Table 1). In addition, some ticks were identified to only the genus level: 10 *Dermacentor* spp., 4 *Rhipicephalus* spp., 18 *Ixodes* spp., 1 *Amblyomma* sp., 1 *Argas* sp., and 1 *Haemaphysalis* sp. Most tick bites (75%) were reported between the months of April and August, and the peak level (19%) was recorded in August. However, the seasonality of tick bites was different for different tick species. *D. marginatus* bites occurred during spring and autumn, whereas *R. sanguineus* bites occurred in summer, with a peak in August. *Ixodes* tick bites were observed between December and October, most frequently occurring between April and August with a peak in June.

3.2. Bacteria detected in ticks

Bacterial DNA was detected in 101 ticks (40.7%) obtained from 86 individuals (Table 2). Among the 67 *Rhipicephalus* ticks, 11 were