A 4-year study of *Anaplasma phagocytophilum* in Portugal

A. S. Santos¹, F. Bacellar¹ and J. S. Dumler²

¹Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Dr Ricardo Jorge, Lisboa, Portugal and ²Division of Medical Microbiology, Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, MD, USA

**INTRODUCTION**

*Anaplasma phagocytophilum* has traditionally been regarded as a worldwide veterinary tick-borne bacterium, and more recently as an emerging human pathogen causing human granulocytic anaplasmosis (HGA) [1]. The growing interest in this agent in Europe [2], along with its detection in Portugal, has signalled the need for detailed study that addresses the ecobiology and disease occurrence in our country. Using serological, molecular and/or isolation attempts, we studied potential *A. phagocytophilum* infections in ticks, rodents, horses, dogs and humans. The present work summarises the main results obtained during a 4-year study started in 2002.

**MATERIAL AND METHODS**

**Sample in study**

A total of 2006 ticks belonging to 14 species (67% *Ixodes*) collected in seven mainland districts and on Madeira Island were studied by PCR. Serum and/or tissue samples from 322 rodents, including five different species collected in four mainland districts and on Madeira Island, 302 horse sera from 10 mainland districts, and 55 EDTA-blood samples from dogs with suspected tick-borne disease from one mainland district, were studied by IFA and PCR. Sera from 792 clinically ill individuals submitted to CEVDI/INSA for the laboratory diagnosis of tick-borne disease during 2000–2006 were also studied by IFA for *A. phagocytophilum*. Investigation of active infection by PCR and in vitro isolation were performed on whole-blood samples submitted in 2006. Positive sera were also screened by PCR to detect *A. phagocytophilum* DNA.

Serology

Antibodies against *A. phagocytophilum* were detected by indirect immunofluorescent assay (IFA) using commercial (Focus Diagnostics, Cypress, California) or in-house antigen slides prepared with *A. phagocytophilum* Webster strain-infected HL-60 cells. Antibody classes examined included IgM and IgG or total polyvalent immunoglobulins. A sample with an IFA titre ≥80 was interpreted as positive and then serially diluted to end-point titre.

Molecular testing

*Anaplasma phagocytophilum* DNA was screened by nested or single tube PCR reactions using different sets of primers: (i) GE9f/GE10r or GE3a/GE10r followed by GE9f/GE2, which amplify fragments of rrs (16S rRNA gene) [3]; (ii) HS1/H56 followed by HS43/H545 derived from the heat-shock operon (groESL) [4]; and (iii) Msp465f/Msp980r targeting the highly conserved 5’ region of *msp2* (major surface protein-2) paralogs [5]. The identity of amplified DNA was confirmed by sequencing.

Isolation attempts

Samples were inoculated into HL-60 cells maintained in antibiotic-free RPMI-1640 medium, supplemented with 2 mM L-glutamine and 5% fetal bovine serum (Gibco, Invitrogen™, Scotland, UK), and incubated at 37°C in 5% CO₂. Microbial growth was checked every 2–4 days by microscopy of cytocentrifuged culture aliquots stained with Diff-Quik (Medion Diagnostics GmbH, Düdingen, Switzerland).

**RESULTS AND CONCLUSIONS**

Two *Ixodes* species harbored *A. phagocytophilum*, including 6/142 (4.2%) *Ixodes ricinus* on Madeira Island, and 3/144 (2.1%) *I. ventralloi* collected on the mainland. The detection of *A. phagocytophilum* DNA in *I. ricinus* reinforced prior studies suggesting its persistence on Madeira Island. This work also provided the first evidence of infections in *I. ventralloi* ticks. Because some *I. ventralloi* infest cats, both could be complicit in enzootic maintenance on the mainland. Moreover, sequencing data provided evidence of *A. phagocytophilum* variant genotypes in Portuguese ticks. Partial...
gene sequences from infected ticks demonstrated nucleotide polymorphisms supporting a close relationship of *A. phagocytophilum* on Madeira Island *I. ricinus* (sequence type PoTiA1dt) to genotypes detected in Central and Northern Europe as well as North American strains isolated from humans, such as the Californian strains CAHU-HGE1 and CAHU-HGE2. Yet, these variants diverge from those found on mainland *I. ventalloi* (sequence type PoTiA2dt), which possibly represents a new genotype of undetermined pathogenicity. When compared with the closest sequence types, CAHU-HGE1 and CAHU-HGE2, *rrs* and *groESL* sequences were 100% similar for PoTiA1dt and 100% and 97.8% for PoTiA2dt, respectively.

IFA-positive results were detected in 7/194 (3.6%) *Mus spretus*, 9/302 (3%) horses and 30/55 (55%) dogs from the mainland. PCR testing of samples from these animals provides the first definitive evidence of *A. phagocytophilum* active infection in Portuguese vertebrates. DNA exhibiting *rrs* and *groESL* nucleotide sequence similarities of 98.9% and 99.7%, respectively, to the human *A. phagocytophilum* HZ strain were found in one seropositive horse from mainland Portugal (sequence type PoAnA1dt), suggesting the potential for HGA in Portugal. *Anaplasma platys* DNA was also identified in *A. phagocytophilum*-seropositive dogs, suggesting serological cross-reactions.

Studies of human samples received for diagnosis of suspected tick-borne disease showed *A. phagocytophilum* seropositivity in 31/792 (3.9%), including two cases fulfilling diagnostic criteria, although neither were PCR- or isolation-confirmed. Moreover, some seropositive patients had additional evidence of other tick-borne agents or related bacterial infections, including Lyme borreliosis (*n* = 2), Q fever (*n* = 7) and bartonellosis (*n* = 1). Although false positive cross-reactions to shared antigens have not been excluded, these reactions could potentially be the result of active dual infections, or past exposure to several agents transmitted by *Ixodes* species. Overall, these results argue for continued improvements in *A. phagocytophilum* diagnostics, especially direct detection techniques, as well as integrated analysis of diagnostic tests for patients with suspected *Ixodes*-borne disease.

**ACKNOWLEDGEMENTS**

This research was partially supported by the Portuguese government through the Fundação para a Ciência e a Tecnologia grant BD/8610/2002.

**REFERENCES**


