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E. PAPADOGIANNAKIS (Ε. ΠΑΠΑΔΟΓΙΑΝΝΑΚΗΣ),
G. SPANAKOS (Γ. ΣΠΑΝΑΚΟΣ), I. KONTOU (Ι.
ΚΟΝΤΟΥ), V. KONTOS (Β. ΚΟΝΤΟΣ), E. VELONAKIS
(Ε. ΒΕΛΟΝΑΚΗΣ), D. SKLIVAS (Δ. ΣΚΛΗΒΑΣ), C.
KOUTIS (Χ. ΚΟΥΤΗΣ)

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■ First identification of *Bartonella coopersplainsensis* in wild rodents (*Rattus norvegicus*) in Greece.

Papadogiannakis E.^a DVM, CertSAD, PhD, Spanakos G.^b PhD, Kontou I.^a MD, MPH, Kontos V.^a DVM, PhD, Velonakis E.^c MD, PhD, Sklivas D.^a MPH, Koutis C.^d MD, PhD

^a Department of Veterinary Public Health, National School of Public Health, Athens, Greece

^b Department of Parasitology, Entomology and Tropical Diseases, National School of Public Health, Athens, Greece

^c Department of Microbiology and Applied Immunology, National School of Public Health, Athens, Greece

^d Department of Public Health, Technological Educational Institution of Athens, Athens, Greece

■ Η ταυτοποίηση της *Bartonella coopersplainsensis* σε άγρια τρωκτικά (*Rattus norvegicus*) για πρώτη φορά στην Ελλάδα.

Ε. Παπαδογιαννάκης^a DVM, CertSAD, PhD, Γ. Σπανάκος^b PhD, Ι. Κοντού^a MD, MPH, Β. Κοντός^a DVM, PhD, Ε. Βελονάκης^c MD, PhD, Δ. Σκλήβας^a MPH, Χ. Κουτής^d MD, PhD

^a Τομέας Κτηνιατρικής Δημόσιας Υγείας, Εθνική Σχολή Δημόσιας Υγείας, Αθήνα

^b Τομέας Παρασιτολογίας, Εντομολογίας και Τροπικών Νοσημάτων, Εθνική Σχολή Δημόσιας Υγείας, Αθήνα

^c Τομέας Μικροβιολογίας και Εφαρμοσμένης Ανοσολογίας, Εθνική Σχολή Δημόσιας Υγείας, Αθήνα

^d Τομέας Δημόσιας Υγιεινής, Τ.Ε.Ι. Αθήνας

ABSTRACT. This study was a preliminary attempt to detect *Bartonella* sp. in tissues (liver and heart) obtained from wild rodents (*Rattus norvegicus*) in central Greece. Eighteen (18) liver and eighteen (18) heart samples were examined, which were obtained from twenty three (23) animals. Two (2) liver samples were found positive, while the heart samples examined were found negative for *Bartonella* sp. 16S-23S intergenic spacer rDNA gene by PCR. Sequencing of the positive PCR products and comparison with those available in GenBank using the BLAST program revealed the same species of *Bartonella* in both positive samples with 100% sequence homology to *Bartonella coopersplainsensis*.

Keywords: *Bartonella coopersplainsensis*, *Rattus norvegicus*, Greece, PCR

ΠΕΡΙΛΗΨΗ. Η μελέτη αυτή ήταν μία πρώτη προσπάθεια για ανίχνευση της *Bartonella* sp. σε ιστούς (ήπαρ και καρδιά) που πάρθηκαν από άγρια τρωκτικά (*Rattus norvegicus*) στην κεντρική Ελλάδα. Εξετάστηκαν δεκαοκτώ (18) δείγματα ήπατος και δεκαοκτώ (18) δείγματα καρδιάς, τα οποία προέρχονταν από είκοσι τρία (23) ζώα. Δύο (2) δείγματα ήπατος βρέθηκαν θετικά ενώ όλα τα δείγματα καρδιάς ήταν αρνητικά για το γονίδιο 16S-23S intergenic spacer rDNA της *Bartonella* sp. Η αλληλουχία των βάσεων του DNA των θετικών δειγμάτων και η σύγκριση με τα διαθέσιμα δεδομένα της GenBank με τη χρησιμοποίηση του προγράμματος BLAST, αποκάλυψε το ίδιο είδος *Bartonella* και στα δύο θετικά δείγματα με 100% ομοιότητα με την αλληλουχία των βάσεων της *Bartonella coopersplainsensis*.

Λέξεις ευρετηρίασης: *Bartonella coopersplainsensis*, *Rattus norvegicus*, Ελλάδα, PCR

Correspondence: Papadogiannakis E.

Department of Veterinary Public Health, National School of Public Health, 115 21 Athens, Greece
Tel.: +030 2132010217, Fax.: +030 2109818444, E-mail: dermpap1@otenet.gr

Αλληλογραφία: Ε. Παπαδογιαννάκης

Τομέας Κτηνιατρικής Δημόσιας Υγείας, Εθνική Σχολή Δημόσιας Υγείας,

Λ. Αλεξάνδρας 196, 115 21 Αθήνα

Τηλ.: +0302132010217, Fax.: +0302109818444, E-mail: dermpap1@otenet.gr

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The genus *Bartonella* includes fastidious, Gram-negative, aerobic, facultatively intracellular bacilli of erythrocytes and endothelial cells of various mammals. A significant number of *Bartonella* species is considered zoonotic pathogens and, apart from humans, they can infect several animal species including rodents, ruminants, cats and dogs (Breitschwerdt and Kordick 2000). In addition, *Bartonella* species have been detected in several arthropod vectors like fleas, lice and ticks (Billeter et al. 2008). It has been reported that *Bartonella* species are widely distributed among rodents in southern China (Ying et al. 2002), Thailand (Castle et al. 2004), Bangladesh (Bai et al. 2007), Europe (Birtles and Harrison 1994, Ellis et al. 1999, Engbaek and Lawson 2004) and America (Kosoy et al. 1997, Ellis et al. 1999). There has been only one report in which three *Bartonella* strains have been detected in wild rodents in northern Greece (Tea et al. 2004).

This study was a preliminary attempt to detect *Bartonella* sp. in tissues (liver and heart) obtained from wild rodents (*Rattus norvegicus*) in Greece.

All animals were live trapped at randomly selected suburban areas in the cities of Athens and Piraeus (it is the greater Athens area) in central Greece. Wire mesh traps were put from 6 p.m to 7 a.m.

Upon arrival at the Molecular Biology Laboratory of the National School of Public Health, the rodents were given general anaesthesia using a combination of xylazine and ketamine intramuscularly. Under aseptic conditions, the liver and heart were removed and the animal was then euthanized. A small part of the excised tissues was placed in sterile eppendorf tubes and stored at -80°C until DNA isolation. Samples were handled under sterile conditions to avoid the risk of cross-contamination. Liver and heart are among the internal organs affected following experimental inoculation of *Bartonella* sp. in immunocompetent mice (Colton et al. 2010).

In total, 18 heart and 18 liver samples were examined, which were obtained from 23 animals. All rodents were adult and belonged to the species *Rattus norvegicus*. Thirteen were males (56.52%) and 10 were females (43.48%). In 13 animals, the heart and liver samples were obtained from the same animal.

DNA was isolated from approximately 10 mg of liver or heart tissue, using the QIAamp DNA Mini kit (Qiagen, Valencia, CA, USA). DNA isolation was

performed according to the manufacturer's recommendations and DNA was eluted in 100 µl elution buffer (EB buffer) for increased concentration. Detection of *Bartonella* DNA by PCR was performed at 25 µl total reaction volume containing 1x PCR buffer, 2 mM Magnesium Chloride, 0.2 mM of each dNTP 1 µM of each primer and 5 µl of template DNA. Thermal cycling was performed with the following conditions: an initial denaturation step at 94°C for 2 min was followed by 45 cycles of 94°C for 15 sec, 66°C for 15 sec and 72°C for 15 sec and a final elongation step at 72°C for 10 min. Primers used were

325s:

(5CTTCAGATGATGATCCCAAGCCTTCTGGCG) and

1100as:

(5GAACCGACGACCCCCTGCTTGCAAAGCA3),

obtained from Syntezza (Israel), as previously described (Maggi et al. 2006). These primers amplify a region of 16S-23S ribosomal RNA intergenic spacer (ITS) (Cadenas et al. 2008, Maggi et al. 2006). One positive (DNA extracted from *Bartonella henselae* from culture) and one negative control were included in each reaction. PCR products were electrophorised in 2% agarose gel containing ethidium bromide, visualized under UV light and photographed with a digital camera. The band of interest was excised and DNA was isolated using the QIAquick gel extraction kit (Qiagen, Valencia, CA, USA) according the manufacturer's instructions. Both strands of the DNA were directly sequenced at Biogenomica (Vrillisia, Athens, Greece) using the primers used for PCR. Nucleotide sequences were manually corrected using finchty 1.3.1 for linux (downloaded from <http://www.geospiza.com/Products/finchty.shtml>). Sequences obtained were compared to those available in GenBank using the web interface of BLAST 2.2.23+ program (Zhang et al. 2000).

Two (2) liver samples were found positive for *Bartonella* sp. by PCR, representing a prevalence of 11.11% of the total number of liver samples and 8.69% of the total number of animals examined. In contrast, all heart samples examined were found to be negative. A heart sample was available and examined in only one out of the two positive animals. Sequencing of the PCR products obtained in two independent reactions and comparison with those available in GenBank using the

BLAST program revealed sequences identical to each other (GenBank accession number HM749323), with 100% sequence homology to *Bartonella coopersplainsensis* strain AUST/NH20 (EU111770), *Bartonella* sp. strain Rt222sm (AY277896) and *Bartonella* sp. strain Lao/Nh1 (EU714976).

Rodents seem to play an important role in *Bartonella* infection, since several species of *Bartonella* have been detected in rodents by isolation and/or molecular detection. It has been shown that a) rodents constitute a natural “reservoir” of various species of *Bartonella*, b) the bacterium is commonly isolated from rodents living in suburban areas and c) following experimental infection congenital transmission has been observed (Ellis et al. 1999). Accordingly, the determination of the *Bartonella* species in wild rodents in a specific area (or country) greatly contributes to the better understanding of the epidemiology and prevention of *Bartonella* spreading to animals and humans.

There has been only one published report in which *Bartonella taylorii*, *Bartonella birtlesii* and *Bartonella grahamii* were isolated from the blood of wild rodents (*Apodemus flavicollis*, *Microtus agresti* and *Dryomys nitedula*) in northern Greece (Tea et al. 2004). However, to the best of the authors’ knowledge, *Bartonella coopersplainsensis* identified in the present study has never been reported either in Greece or in Europe until today. It seems that different species of rodents host different *Bartonella* species, although different geographical distribution of the bacterium cannot be excluded.

Bartonella coopersplainsensis isolated from the blood of wild rats of the species *Rattus leucopus* in Australia has recently been identified as a novel *Bartonella* species (Gundi et al. 2009).

It has been shown that *Bartonella* sp. has been detected in the liver and heart of experimentally inoculated mice (Colton et al. 2010). This probably explains the detection of *Bartonella* in the liver samples in our study, although the heart sample was not

available from one positive animal. In a recent study carried out in Japan, bacteria belonging to *Bartonella* species were not detected in the blood obtained from *Rattus norvegicus* (Inoue et al. 2008), whereas a similar study in southern France revealed a 16.6% infection in the blood and heart samples obtained from *Rattus norvegicus* (Gundi et al. 2004).

The zoonotic potential of rodent-borne *Bartonella* spp. is emerging, as suggested by a novel rodent *Bartonella*-associated febrile disease in the rural southwestern USA, based on serological evidence (Iralu et al. 2006). At present, only a limited number of human infections caused by rodent-borne *Bartonella* species has been reported. However, previous experience has shown that *Bartonella* species firstly detected or isolated in animals could be later involved in humans, such as *Bartonella alsatica*, which was firstly isolated from wild rabbits (Heller et al. 1999) and later identified in cases of endocarditis (Raoult et al. 2006) and cat scratch disease (Angelakis et al. 2008) in humans. Accordingly, although the public health significance of *Bartonella coopersplainsensis* identified in our study remains to be clarified, it should be prudent for people working in conditions bringing them in contact with wild rodents to take the appropriate measures to protect themselves from direct contact with these animals, as well as their ectoparasites (e.g. fleas). Arthropods (ectoparasites) constitute one of the main ways of *Bartonella* transmission from one animal to another or to humans (Morick et al. 2009).

Further studies are needed to clarify in which way the wild rodents *Rattus norvegicus* contaminate the environment with *Bartonella coopersplainsensis* and possible mode of transmission to humans and other animals.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper. ■

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