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Molecular detection of *Bartonella* spp. in humans, domestic animals and ectoparasites sampled in Lisbon, Portugal.

Estefania Ysabel Torrejón Navarro

DISSERTAÇÃO PARA A OBTENÇÃO DO GRAU DE MESTRE EM CIÊNCIAS BIOMÉDICAS

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Resumo

Deteção molecular de *Bartonella* spp. em humanos, animais domésticos e ectoparasitas amostrados em Lisboa, Portugal.

Palavras-chave: *Bartonella*, Portugal, carraças, gatos, cães, humanos.

Bartonella spp. compreende pequenas bactérias fastidiosas com distribuição mundial. São consideradas agentes patogénicos zoonóticos negligenciados, cuja transmissão ao ser humano ocorre através do contato com mamíferos infetados e artrópodes que se alimentam de sangue. Os gatos são o principal reservatório e hospedeiro de pelo menos três *Bartonella* spp.: *B. henselae* e *B. clarridgeiae*, ambos agentes causadores da doença da arranhadura do gato, e *B. koehlerae*, agente de endocardite em humanos; adicionalmente, os cães representam um importante reservatório, especialmente para *B. vinsonii* subsp. *berkhoffii*. Embora as pulgas desempenhem um papel importante na transmissão das *Bartonella* spp. felina, outros potenciais vetores artrópodes foram identificados para a presença de DNA de *Bartonella* sp. Desta forma, as carraças representam um grupo importante para a identificação molecular de *Bartonella* sp.. Considerando que existem poucos estudos que abordam a ocorrência de *Bartonella* em Portugal, o presente trabalho teve como foco a identificação e caracterização molecular de *Bartonella* spp. em gatos e cães vadios, humanos e em carraças recolhidas no país referido. As recolhas de carraças foram realizadas usando o método da bandeira de arrasto de 2012-2018 em 19 pontos geográficos organizados em 8 distritos do continente. Para este estudo, foram doados 400 microlitros de sangue total em EDTA de cães e gatos não domiciliados que participavam do programa de esterilização promovido pela Casa dos Animais de Lisboa (CAL) e 1000 µl de sangue de indivíduos que estiveram em contato com gatos, recolhido após consentimento prévio. O DNA de gatos, cães e humanos foi extraído e a integridade do DNA confirmada por PCR convencional direcionado para os genes endógenos *GAPDH* e 18SRNA para mamíferos e carraças, respetivamente. A deteção e quantificação molecular foram realizadas por qPCR visando um fragmento do gene *nuoG*. As amostras positivas foram caracterizadas por PCR convencional para os genes de *Bartonella* spp. *gltA* e *ribC*. Das 123 amostras de sangue de gato, 25 (20.32%) foram positivas (quantificação) para o gene referido; destas amostras positivas, 13 foram caracterizadas como *Bartonella henselae*, 11 como *B. clarridgeiae* e 1 apresentaram co-infecção de ambas as espécies. A quantificação absoluta do DNA *nuoG* da *Bartonella* sp. em gatos amostrados variou de $2,78 \times 10$ a $1,03 \times 10^5$ cópias / µL. Pelo contrário, das 25 amostras de sangue de cães, 30 amostras de sangue humano e 236 amostras de carraças adultas (154 *Ixodes* spp. e 82 *Rhipicephalus sanguineus* sensu lato), todas foram negativas para o gene *nuoG*.

Os resultados obtidos sugerem que as espécies *B. henselae* e *B. clarridgeiae* circulam em gatos vadios mas nenhum cão, humano ou carraça apresentaram resultados positivos para o agente patogénico. Apesar de nossos resultados não sugerirem os cães e humanos como reservatórios e hospedeiros de *Bartonella* spp., respectivamente, e as carraças como vetores competentes, mais estudos usando novas abordagens precisam de ser realizados para clarificar o seu papel na transmissão de *Bartonella* spp.

Abstract

Molecular detection of *Bartonella* spp. in humans, domestic animals and ectoparasites sampled in Lisbon, Portugal.

Key words: *Bartonella*, Portugal, ticks, cats, dogs, humans.

Bartonella spp. comprises fastidious gram-negative bacteria with worldwide distribution. They are considered neglected zoonotic pathogens, whose transmission to humans occurs through contact with infected mammals and blood-sucking arthropod. Cats are the main reservoir host for at least three *Bartonella* spp.: *B. henselae* and *B. clarridgeiae*, both causative agents for cat scratch disease, and *B. koehlerae* agent for endocarditis in humans, in addition, dogs represent an important reservoir, especially for *B. vinsonii* subsp. *berkhoffii*. Although fleas play a major role in transmission of feline *Bartonella*, other potential arthropod vectors have been identified to harbor *Bartonella* DNA. In this way, ticks represent an important group for *Bartonella* molecular identification. Considering that there are few studies addressing the occurrence of *Bartonella* in Portugal, the present study is focused on the molecular identification and characterization of *Bartonella* spp. circulating in stray cats and dogs, humans and in questing ticks collected in the referred country. Tick collections were performed using the drag-flag method from 2012-2018 in 19 geographical points organized in 8 districts of mainland Portugal. Four hundred microliters of EDTA whole blood from non-domiciled cats and dogs participating in the sterilization program promoted by Casa dos Animais de Lisboa (CAL) was donated for the purpose of this study and 1000 µl human blood was taken after prior consent from individuals that have been in contact with cats. DNA from cats, dogs and humans was extracted and DNA integrity was confirmed by a conventional PCR targeting the endogenous *GAPDH* and 18SRNA genes, for mammals and ticks, respectively. Molecular detection and quantification were carried out by qPCR targeting a fragment of *nuoG*. Positive samples were characterized by a conventional PCR for the *gltA* and *ribC* *Bartonella* spp. genes. From 123 cat blood samples, 25 (20.32%) were positive (quantification) for the referred *Bartonella* gene; from those positive samples, 13 were characterized as *B. henselae*, 11 as *B. clarridgeiae* and 1 presented a co-infection of both species. Furthermore, the absolute quantification of *nuoG* *Bartonella* DNA in sampled cats ranged from 2.78×10 to 1.03×10^5 copies/µL. On the contrary, from 25 dog blood samples, 30 human blood samples, and 236 adult tick samples (154 *Ixodes* spp. and 82 *Rhipicephalus sanguineus* sensu lato), all were negative for the *nuoG* gene.

These results suggest the species *B. henselae* and *B. clarridgeiae* are circulating in stray cats but, in contrast, no dogs, humans or ticks were positive for the pathogen. Despite our results were not supportive regarding dogs and humans as *Bartonella* spp. reservoirs and hosts, respectively, and ticks as competent vectors, more studies using new approaches need to be performed in order to clarify their role in *Bartonella* spp. transmission.

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Abbreviations

μL:	Microliter
μM:	Micromolar
16S rDNA:	16S ribosomal DNA
16S rRNA:	16S ribosomal RNA
18S rRNA:	18S ribosomal RNA
AIDS:	Acquired immune deficiency syndrome
BAPGM:	<i>Bartonella</i> Alpha Proteobacteria Growth Medium
bp:	Base pair
cPCR:	Conventional PCR
dA:	Single adenine nucleotide
DNA:	Deoxyribonucleic acid
EDTA:	Ethylene diamine tetra acetic acid
F:	Forward
<i>ftsZ:</i>	Filamenting temperature-sensitive mutant Z
g:	Gram
<i>GAPDH:</i>	Glyceraldehyde 3-phosphate dehydrogenase
<i>gltA:</i>	Citrate synthase
<i>groEL:</i>	Chaperonin GroEL
<i>IGS:</i>	Intergenic spacer region
<i>ITS:</i>	Internal transcribed spacer
LB:	Luria Bertani
M:	Molar

mL:	Millilitre
mM:	Milimolar
NADH:	Nicotinamide adenine dinucleotide
ng:	Nanograms
nm:	Nanometer
nuoG:	NADH-quinone oxidoreductase subunit G
°C:	Celsius degree
<i>pap31</i>:	Hemin-binding protein
PBS:	Phosphate-buffered saline
PCR:	Polymerase chain reaction
q.s.p.:	Quantum sufficit
qPCR:	Real time PCR
R:	Reverse
<i>ribC</i>:	Riboflavin synthase
RNA:	Ribonucleic acid
rpm:	Revolutions per minute
spp.:	Species (plural)
UV:	Ultraviolet
V:	Volt
W:	Watt

1. Introduction

1. Introduction

1.1 The genus *Bartonella*

The genus *Bartonella* comprises small, facultative, fastidious gram-negative intracellular bacteria with worldwide distribution (Breitschwerdt et al., 2010). They belong to the alpha-2 class of Proteobacteria, order Rhizobiales and family Bartonellaceae (Chomel et al., 2009).

For a long time, the genus *Bartonella* was recognized for presenting only one species, known as *Bartonella bacilliformis*, a pleomorphic (coccoid, cocobacillary or bacillary) agent of Carrion's disease, reported in patients with Oroya Fever and Peruvian wart, an endemic disease to high-altitude valleys of the South Andes (Kuykendall, 2006; Minnick et al., 2014). However, following on molecular studies of DNA sequences from the 16S rRNA subunit, Brenner et al. (1993) observed phylogenetic relationships between *Rochalimaea quintana* and *B. bacilliformis*. Therefore, the transfer of *R. quintana*, belonging to the family Rickettsiaceae, to the family Bartonellaceae was proposed, maintaining the two species in the genus *Bartonella*. With this, four species of the genus *Rochalimaea* were renamed: *B. quintana*, *B. vinsonii*, *B. henselae* and *B. elizabethae* (Chomel et al., 2009).

Currently, there are 45 species in the *Bartonella* genus described and isolated from humans, domestic animals and wild animals (Okaro et al., 2017). Additionally, numerous species and subspecies has been constantly proposed, expanding the genus rapidly (Okaro et al., 2017). Out of this 45 species, at least fifteen are associated with human infections (Breitschwerdt et al., 2010). Each *Bartonella* spp. appears to have co-evolved with a specific primary reservoir host, which poses a source of infection for accidental hosts under natural exposure conditions (Breitschwerdt et al., 2017). For example, *B. henselae* that has co-evolved with cats is the most frequent species identified from humans, as well as from companion animals such as cats and dogs (Chomel et al., 2006 and Deng et al., 2012). Moreover, new reservoirs have been reported, including bats (Corduneau et al., 2018), rodents (Gutiérrez et al., 2015), marine animals (Maggi et al., 2008), terrestrial herbivores such as camels (Ereqat et al., 2016), and wild carnivores, including lions, bears and foxes (Bai et al., 2016). Infections in human beings by being in contact with these new reservoirs were not reported.

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Bartonella spp. are re-emergent and neglected zoonotic pathogens that infect erythrocytes and endothelial cells, besides macrophages, monocytes and dendritic cells (Musso et al., 2001) (**Figure 1**). Furthermore, they are able to invade and survive in said erythrocytes and endothelial cells, resulting in a long-lasting intraerythrocytic and presumably intraendothelial infection, usually linked to a relapsing pattern of bacteremia (Pitassi et al., 2010). This prolonged bacteremia enhance the chance of the pathogen transmission by biological and/or mechanical vectors to new susceptible hosts (Breitschwerdt et al., 2010). The infection is often asymptomatic in their hosts, but in some cases, it can evolve to more severe clinical symptoms (Regier et al., 2016). In most cases, the presence of *Bartonella* spp. in the blood of the infected reservoirs does not result in serious disease, being often asymptomatic, however, these infected animals act as ubiquitous reservoirs for potential zoonotic infection, where the disease can evolve to more severe clinical symptoms, such as endocarditis and neurological dysfunctions (Regier et al., 2016; Okaro et al. 2017).

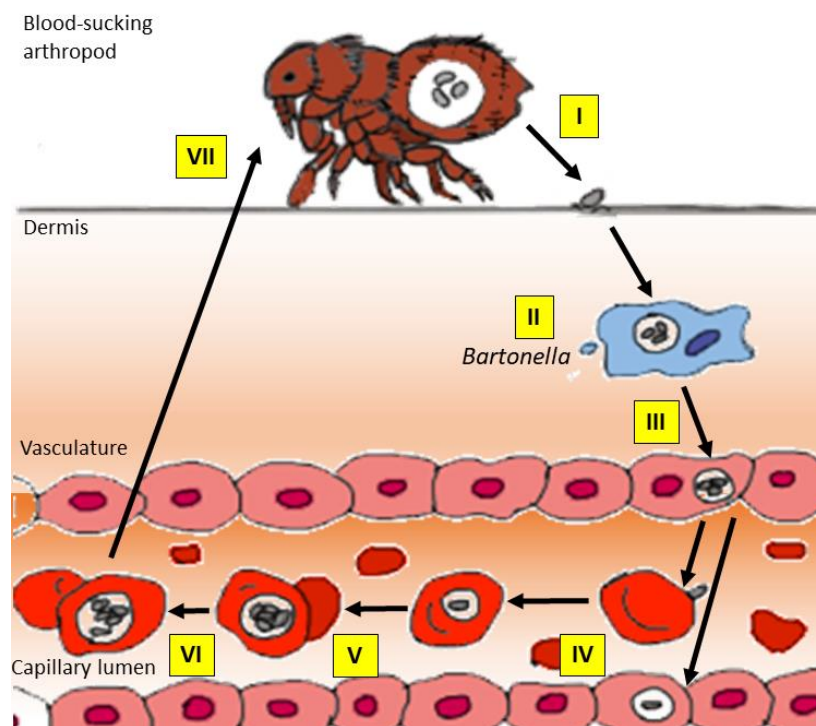


Figure 1. Common infection strategy of *Bartonella* sp. The drawing illustrates the general concept of reservoir host infections with *Bartonella* sp. Following transmission by an arthropod vector (I), *Bartonella* sp. colonize the primary niche, which probably involves

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entry into migratory cells (II) and transport to the vascular endothelium (III), where the bacteria persist intracellularly. From the primary niche, the bacteria are seeded into the bloodstream (IV), where invade erythrocytes and reinfect the primary niche. After limited replication inside the red blood cell (V), bacteria persist in the intraerythrocytic niche (VI) competent for transmission by a bloodsucking arthropod (VII) (Adapted from Harms and Dehio, 2012).

1.2 Human bartonellosis

Human infections caused by several *Bartonella* spp., subspecies and *Candidatus* have been reported, however, the vast majority of infections in humans are attributed to *Bartonella quintana* and *Bartonella bacilliformis*, acquired by infection through an arthropod vector, and to *B. henselae*, acquired by mechanical infection because of contact with a reservoir (Dehio et al., 2004). The signs and symptoms of human bartonellosis generally occur about one to two weeks after exposure, but in some cases, it can range from 1-4 weeks. The symptoms varies with the *Bartonella* spp., specific tissue infected and status of the host immune system (Dehio et al., 2004; Rolain et al., 2004). Immunocompromised hosts, particularly those infected with HIV are more susceptible to infection with *Bartonella* spp. and the clinical course may be more severe (Resto-Ruiz et al., 2003). The clinical spectrum of *Bartonella* sp. infection is expanding, and includes, besides classical diseases such as Carrion's (Chamberli et al., 2002), cat-scratch disease (Maggi et al., 2013; Oksi et al., 2013; Slater et al., 1992), and others such as chronic lymphadenopathy (Oksi et al., 2013), trench fever (Ohl & Spach, 2000), chronic bacteraemia (Maggi et al., 2013; Ohl & Spach, 2000; Slater et al., 1992), culture-negative endocarditis (Daly et al., 1993; Iannino et al., 2018; Oliveira et al., 2010; Maggi et al., 2013; Slater et al., 1992), bacillary angiomatosis (Maggi et al., 2013; Ohl & Spach, 2000; Slater et al., 1992), bacillary peliosis (Maggi et al., 2013; Slater et al., 1992), vasculitis (Balakrishnan et al., 2016), and uveitis (Rothova et al., 1998). Moreover, *Bartonella* sp. infections have also been recently linked to different manifestations such as hallucinations, weight loss, muscle fatigue, partial paralysis, pediatric acute-onset neuropsychiatric syndrome (PANS), and other neurological manifestations (Balakrishnan et al., 2016; Breitschwerdt et al., 2011; Breitschwerdt et al., 2019). A few case studies

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have also documented *Bartonella* sp. in tumors, particularly vasoproliferative and those of mammary tissue (Markaki et al., 2003; Marques et al., 2018; Povoski et al., 2003).

The list of species, subspecies and *Candidatus* species of the genus *Bartonella* already identified by molecular methods in humans, followed by its clinical outcomes is presented in **Table 1**.

Table 1. Species, subspecies, or *Candidatus* species of the genus *Bartonella* detected by molecular methods in symptomatic humans. (Adapted from Viera-Damiani et al., 2015).

Species	Clinical outcome(s)	Reference(s) or source
<i>B. bacilliformis</i>	Carrion Disease (also known as Oroya fever, verruga peruana).	Chamberli et al., 2002
<i>B. quintana</i>	Bacillary angiomatosis, trench fever, bacteremia.	Ohl & Spach, 2000
<i>B. henselae</i>	Cat scratch disease, endocarditis, bacillary angiomatosis, peliosis hepatis, bacteremia, vasculitis, uveitis.	Balakrishnan et al., 2016; Iannino et al., 2018; Maggi et al., 2013; Rothova et al., 1998; Slater et al., 1992
<i>B. elizabethae</i>	Fever, endocarditis.	Daly et al., 1993
<i>B. grahamii</i>	Cat scratch disease (lymphadenopathy, reddish papule, abscess).	Oksi et al., 2013
<i>B. vinsonii</i> subsp. <i>Berkhoffii</i>	Endocarditis, arthritis, neurological disease, and epithelioid hemangioendothelioma.	Oliveira et al., 2010
<i>B. koehlerae</i>	Anxiety, severe headaches, muscle spasms, decreased peripheral vision, and hallucinations.	Breitschwerdt et al., 2011
<i>B. alsatica</i>	Fever, lymphadenitis, and endocarditis.	Raoult et al., 2006
<i>B. vinsonii</i> subsp. <i>Arupensis</i>	Fatigue, headache, and myalgias.	Welch et al., 1999
<i>B. washoensis</i>	Meningitis.	Probert et al., 2009
<i>B. rochalimae</i>	Bacteremia, fever, and splenomegaly.	Eremeeva et al., 2007
<i>B. tamiae</i>	Fever, headache, myalgia, maculopapular rash, petechial rash in arms and legs, abnormalities in liver function.	Kosoy et al., 2008
<i>B. melophagia</i>	Dry cough, fatigue, muscle pain, and severe chills.	Maggi et al., 2009
<i>B. mayotimonensis</i>	Endocarditis.	Lin et al., 2010

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Table 1. (continued)

<i>B. volans</i> (including volans-like)	Symptoms uncertain.	Breitschwerdt et al., 2011
<i>B. clarridgeiae</i>	Asymptomatic.	Viera-Damiani et al., 2015

1.3 Vectors

1.3.1 Fleas

Fleas, are small, laterally flattened, wingless, and highly specialized insects that belong to the order Siphonaptera (Pittarate et al., 2018). Both adult males and females are obligate hematophagous ectoparasites of mammals and birds and vessel feeders (Bitam et al., 2010). Their concerning potential as vectors relies on the fact that fleas themselves are hosts to different pathogens, and a natural avenue for *Bartonella* sp. dispersal (Bitam et al., 2010). The two commonly known ways of pathogen transmission by fleas are by oral route through regurgitation of blood meals, or by fecal route, by contaminated fecal pellets (Bitam et al., 2010).

About 2,574 species belonging to 16 families and 238 genera have been described, but only a minority live in association with humans (Lewis, 1993). Although there is no flea specific to humans and only a fraction of them come into contact with them on a regular basis, many fleas are associated with domesticated animals, causing an economic repercussion, rather than an issue with public health (Bitam et al., 2010). Two species of *Ctenocephalides*, *Ctenocephalides felis* (cat flea) and *Ctenocephalides cannis* (dog flea) are the most common in said domestic animals (Bitam et al., 2010). The cat flea can infest cats and dogs in many temperate and tropical regions, but it also infests opossums, raccoons (Pung et al., 1994), and rats (Loftis et al., 2006). They also represent the great majority of fleas in homes (Bitam et al., 2010). The dog flea is closely related to the cat flea; they are very similar in appearance and biology, however, the dog flea is less common on dogs than cat fleas, despite its name (Bitam et al., 2010).

C. felis has been shown experimentally to be a competent vector for transmission of *B. henselae*: After placing presumably infected fleas (collected from bacteraemic cattery cats) on five pathogen-free kittens; within 2 weeks, four of the five kittens were found to be bacteraemic to *Bartonella henselae* (Chomel et al., 1996). DNA of the

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referred pathogen had already been detected in fleas from bacteraemic cats by Koehler et al. (1994). Also, the acquisition of *B. henselae* was demonstrated to occur between three hours after *C. felis* were fed on infected cat blood (Foil et al., 1998). In addition, the bacteria was reported to remain in the flea gut for up to nine days, where bacteria replication also occurred (Foil et al., 1998). In another research, the pathogen transmission was tested via inoculation of infected flea faeces and successfully proved to be transmitted to five cats (Foil et al., 1998). DNA from *Bartonella* sp. was also detected by PCR in flea faeces and cultured faeces generated viable colonies (Higgins et al., 1996). These studies demonstrated that *Bartonella* spp. remain reproductively viable in flea faeces and that transmission to humans (of cat scratch disease in this case) most likely occurs as a result of inoculation of *B. henselae* contaminated flea faeces into the skin via a scratch by a flea-infested cat (Finkelstein et al., 2002). Transmission by cat bites has not been demonstrated yet, but *B. henselae* has already been detected of cat saliva (Oskouizadeh et al., 2010).

C. felis also appears to be a potential vector for *B. clarridgeiae*, *B. quintana*, and *B. koehlerae*, as these *Bartonella* spp. together with *B. henselae* have also been detected by PCR assays targeting *gltA*, *ITS* and *pap31* genes on cats in France (Rolain et al., 2003), being the prevalences of 11.1% for *B. henselae* (9/81), 67,9% for *B. clarridgeiae* (55/81), 17.3% for *B. quintana* (14/81) and 3.7% for *B. koehlerae* (3/81).

The role of other flea species as competent vectors of *Bartonella* spp. has not being extensively studied, but some species with worldwide distribution like *Ctenocephalides canis* and *Pulex irritans* have had reports for *B. henselae* and *B. quintana* presence, respectively (Bitam et al., 2010). Current known records are summarized in **Table 2** for three *Bartonella* sp.-related diseases: cat scratch disease, bacillary angiomatosis and endocarditis.

Table 2. *Bartonella* spp. detected in fleas worldwide. (Adapted from Bitam et al., 2010).

Flea	<i>Bartonella</i> species	Disease in humans	Known geographical distribution region of <i>Bartonella</i> spp.
<i>Ctenocephalides felis</i>	<i>B. henselae</i> , <i>B. clarridgeiae</i> , <i>B. quintana</i> , <i>B. koehlerae</i>	CSD, BA, BAC, END	France, Japan, New Zealand, Thailand, UK, USA, Portugal

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Table 2 (continued).

<i>Ctenocephalides canis</i>	<i>B. henselae</i>	CSD, BA	Japan
<i>Pulex irritans</i> <i>/Pulex simulans</i>	<i>B. quintana</i> , <i>Bartonella</i> sp	BAC, END	Gabon, Peru
<i>Xenopsylla cheopis</i>	<i>B. elizabethae</i> , <i>B. tribocorum</i>	END	Algeria, Egypt
<i>Leptopsylla segnis</i>	<i>Bartonella</i> sp, <i>B. elizabethae</i>	END	Algeria, Egypt
<i>Nosopsyllus fasciatus</i>	<i>Bartonella</i> sp	Unknown	Thailand
<i>Oropsylla hirsuta</i>	<i>Bartonella</i> sp	Unknown	USA (Colorado)
<i>Archeopsylla erinacei</i>	<i>B. clarridgeiae</i> , <i>B. elizabethae</i>	END	Algeria unpublished data
<i>Pulex</i> sp	<i>B. rochalimae</i>		Peru
<i>Ornithophaga</i> sp	<i>Bartonella</i> sp, <i>B. elizabethae</i>	END	Portugal
<i>Stenoponia tripectinata</i>	<i>Bartonella</i> sp	END	Portugal
<i>Ctenophthalmus nobilis</i>	<i>B. taylorii</i> , <i>B. grahamii</i>	END	England
<i>Ctenophthalmus lushniensis</i>	<i>B. clarridgeiae</i>	END	China

(CSD: cat scratch diseases; BA: bacillary angiomatosis; END: endocarditis; BAC: bacteremia).

1.3.2 Ticks

Ticks englobe about 900 species, grouped in three families (Guglielmone et al., 2010). The Ixodidae refers to the so-called ‘hard ticks’ which are morphologically characterized by a sclerotized dorsal scutum, while in the Argasidae (or soft ticks), composed by circa of 200 species, the scutum is absent (Guglielmone et al., 2010). The family Nuttalliellidae is represented by a single species, *Nuttalliella namaqua*, confined to Southern Africa and possess some characteristics found in hard and soft ticks, in all life stages (Latiff et al., 2012). The *Ixodes ricinus* complex belongs to the genus *Ixodes*, which belongs to major family Ixodidae, with 243 species (Guglielmone et al., 2010).

Ticks are relevant vectors for bacterial, viral, and protozoal pathogens in different regions of the world (Karasartova et al., 2018). Because of the capacity for transstadial and transovarial transmission that some species possess when infected with specific

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pathogens, they can act not only as vectors but also as reservoirs of these tick-transmitted agents. (Parola and Raoult, 2001). For example, *Borrelia burgdorferi*, the causative agent for Lyme disease, infects the tick as a larva, and the infection is maintained when the larva molts to a nymph and later develops as an adult (Goodman et al., 2005), in addition, *Rickettsia rickettsii*, carried within ticks, is passed on from parent to offspring tick by transovarial transmission (Murray et al., 2005). Transstadial transmission has already been reported for *Ixodes* sp. ticks infected with *B. henselae* (Chang et al., 2001 and Rar et al., 2005) but transovarial transmission experiments for the same bacteria were inconclusive (Cotté et al., 2008).

There has been considerable interest in investigating the role of ticks as potential vectors for *Bartonella* spp. (Angelakis et al., 2010). In this way, an initial step was to define which species may harbor the bacteria, mainly considering the questing stage (Angelakis et al., 2010). As a consequence, molecular and epidemiology surveys have been carried out to identify *Bartonella* spp. DNA in ticks (Adelson et al., 2004; Billeter et al., 2008; Bogumila et al., 2005; Chang et al., 2002; Eskow et al., 2001; Loftis et al., 2005; Halos et al., 2005; Hercik et al., 2007; Kim et al., 2005; Matsumoto et al., 2008; Morozova et al., 2004; Podsiadly et al., 2007; Rar et al., 2005; Sanogo et al., 2003; Schabereiter-Gurtner et al., 2003; Schouls et al., 1999; Sun et al., 2008; Wikswo et al., 2007). *Bartonella* spp. have mostly been identified by PCR using primers targeting either specific *Bartonella* sp. genes like the citrate synthase gene (*gltA*), the riboflavin synthase gene, the heat shock protein gene (*groEL*), the 16S–23S intergenic spacer, the hemin binding protein gene, the cell division protein gene, and the 16S rDNA gene (Angelakis et al., 2010). An overview of these studies are shown in **Table 3**.

Table 3. Questing ticks in which *Bartonella* spp. DNA has been found. (Adapted from Angelakis et al., 2010).

Tick species	Prevalence of <i>Bartonella</i> spp. DNA in ticks, %/no.	Identified <i>Bartonella</i> spp.	Target gene	Reference
<i>Amblyomma americanum</i>	0.43/466 individuals	<i>Bartonella tamiae</i> -like	<i>IGS</i>	Billeter et al., 2008
<i>Carios kelleyi</i>	3.2/31 individuals	Resembling <i>B. henselae</i>	<i>IGS</i>	Loftis et al., 2005

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Table 3 (continued).

<i>Dermacentor nitens</i>	1/1 pool	<i>B. quintana</i> (100% homology)	<i>nuoG</i>	Billeter et al., 2011
<i>Dermacentor occidentalis</i>	8.3/12 pools	<i>Bartonella</i> spp.	<i>gltA</i>	Chang et al., 2002
<i>Dermacentor reticulatus</i>	21.4/84 individuals	<i>B. henselae</i> (99% homology) and <i>B. quintana</i> (90% homology)	<i>groEL</i>	Rar et al., 2005
<i>Dermacentor variabilis</i>	14.3/ 7 pools	<i>Bartonella</i> spp.	<i>gltA</i>	Chang et al., 2002
<i>Haemaphysalis flava</i>	2.7/74 pools	<i>Bartonella</i> spp.	16S rRNA	Kim et al., 2005
<i>Haemaphysalis longicornis</i>	4.4/1,173 pools	<i>Bartonella</i> spp.; 1 pool harbored <i>B. rattimassiliensis</i> (99.2%), 1 pool harbored <i>B. tribocorum</i> (98.3%)	16S rRNA	Kim et al., 2005
<i>H. longicornis</i>	36/150 groups (60 individual fed adults, 30 pools of 2 unfed adults, and 60 pools of 5 nymphs)	<i>Bartonella</i> spp.	<i>gltA</i>	Sun et al., 2008
<i>Ixodes nipponensis</i>	5.0/20 pools	<i>Bartonella</i> spp.	16S rRNA	Kim et al., 2005
<i>I. pacificus</i>	19.2 of 151 individuals	<i>B. henselae</i> , <i>B. quintana</i> , <i>B. washoensis</i> , <i>B. vinsonii</i> subsp. <i>berkhoffii</i> , and a <i>Bartonella</i> cattle strain	<i>gltA</i>	Chang et al., 2001
<i>I. pacificus</i>	11.6/224 pools	<i>Bartonella</i> spp.	<i>gltA</i>	Chang et al., 2002
<i>I. persulcatus</i>	37.6/125 individuals	<i>B. henselae</i> (99% homology) and <i>B. quintana</i> (90% homology)	<i>groEL</i>	Rar et al., 2005
<i>I. persulcatus</i>	44/50 in 2002 and 38/50 in 2003	<i>B. henselae</i>	<i>groEL</i>	Morozova et al., 2004
<i>I. persulcatus</i>	33.3/3 pools	<i>Bartonella</i> spp.	16S rRNA	Kim et al., 2005

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Table 3 (continued).

<i>I. ricinus</i>	1.48/271 individuals	<i>Bartonella henselae</i>	<i>groEL</i> , <i>pap31</i> , <i>ftsZ</i>	Sanogo et al., 2003
<i>I. ricinus</i>	4.9/102 individuals	<i>Bartonella henselae</i>	<i>gltA</i>	Podsiadly et al., 2007
<i>I. ricinus</i>	60/121 individuals	<i>Bartonella</i> spp	16S rRNA	Schouls et al., 1999
<i>I. ricinus</i>	A pool/12 ticks	<i>Bartonella</i> spp	16S rDNA	Schabereiter-Gurtner et al., 2003
<i>I. ricinus</i>	9.8/92 individuals	<i>Bartonella</i> spp.; 1 adult harbored <i>Bartonella schoenbuchensis</i> (96% homology)	<i>gltA</i>	Halos et al., 2005
<i>I. ricinus</i>	7.7/103 individuals	<i>Bartonella capreoli</i>	<i>ITS</i>	Bogumila et al., 2005
<i>I. ricinus</i>	1.2/327 individuals	<i>Bartonella</i> spp.	16S rRNA	Hercik et al., 2007
<i>I. ricinus</i>		Resembling <i>B. bacilliformis</i> †		Kruszewska et al., 1996*
<i>I. scapularis</i>	2.0/203 individuals	<i>Bartonella schoenbuchensis</i>	<i>gltA</i>	Matsumoto et al., 2008
<i>I. scapularis</i>	34.5/107 individuals	Unidentified <i>Bartonella</i> spp.	16S rRNA	Adelson et al., 2004
<i>I. scapularis</i>		<i>Bartonella henselae</i>	16S rRNA	Eskow et al., 2001
<i>I. sinensis</i>	16.3/86 individuals	<i>Bartonella</i> spp.	<i>gltA</i>	Sun et al., 2008
<i>I. spp.</i>	42.3/26 pools	<i>Bartonella</i> spp.	16S rRNA	Morozova et al., 2004
<i>I. turdus</i>	11.1/9 pools	<i>Bartonella</i> spp.; 1 pool harbored <i>Bartonella doshiae</i> (99.2% homology)	16S rRNA	Kim et al., 2005
<i>Rhipicephalus sanguineus</i>	3.2/62 individuals	<i>Bartonella henselae</i>	<i>ribC</i>	Wikswow et al., 2007
<i>Rhipicephalus sanguineus</i>	1/4 pools	<i>B. quintana</i> , <i>B. elizabethae</i> and <i>B. rochalimae</i>	<i>ITS</i> , <i>nuoG</i>	Billeter et al., 2008

Furthermore, for humans, reported tick attachment has preceded an onset of illness in patients from whom *Bartonella* spp. has been detected by serology and/or molecular methods (Arnez et al., 2003; Breitschwerdt et al., 2007; Breitschwerdt et al., 2008; Eskow

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et al., 2001; Lucey et al., 1992; Podsiably et al., 2003; Zangwill et al., 1993). Cases that suggests ticks might serve as potential *Bartonella* sp. vectors are presented in **Table 4**.

Table 4. Evidence of *Bartonella* spp. infection in persons after tick bite. (Adapted from Angelakis et al., 2010).

Agent	Tick species	Tick bite	Animal contact	Clinical manifestation	Reference
<i>B. henselae</i>	Unknown	Yes	No mentioned	Fever, myalgia, arthralgia, headaches, and light sensitivity	Lucey et al., 1992
<i>B. henselae</i>	Unknown	Yes	Cat	Fever, myalgia, arthralgia, headaches, and light sensitivity	Lucey et al., 1992
<i>B. henselae</i>	Unknown	Yes	Cat and kitten	Cat-scratch disease signs	Zangwill et al., 1993
<i>B. henselae</i> , <i>Borrelia burgdorferi</i>	Possibly <i>Ixodes scapularis</i>	Yes	Not mentioned	Low-grade fever, headaches, fatigue, knee arthralgia, and insomnia	Eskow et al., 2001
<i>B. henselae</i> , <i>B. burgdorferi</i>	Possibly <i>I. scapularis</i>	Yes	Not mentioned	Fever, headache, dizziness, fatigue, and arthralgia	Eskow et al., 2001
<i>B. henselae</i> , <i>B. burgdorferi</i>	Unknown	Not mentioned	Not mentioned	Meningitis	Podsiably et al., 2003
<i>B. henselae</i> or <i>B. quintana</i> seroreactive	Unknown	Yes	Not mentioned	Fever	Arnez et al., 2003
<i>B. burgdorferi</i> , <i>B. henselae</i> , <i>B. quintana</i>	Unknown	Yes	Not mentioned	Fever	Arnez et al., 2003
<i>Bartonella</i> spp. closely related to <i>B. henselae</i> , <i>B. quintana</i>	Unknown	Yes	Not mentioned	Not mentioned	Morozova et al., 2005

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Table 4 (continued).

<i>B. henselae</i> and/or <i>B. vinsonii</i> subsp. <i>berkhoffii</i> ¹	Unknown	Yes	Cats, dogs, potentially other animal species	Fatigue, insomnia, arthralgia, myalgia, headache, and/or tremors	Breitschwerdt et al., 2007
<i>B. henselae</i> , and/or <i>B. vinsonii</i> subsp. <i>berkhoffii</i> †	Unknown	Yes	Cats, dogs, other animal species	Seizures, ataxia, memory loss, tremors, fatigue, and/or headaches	Breitschwerdt et al., 2008

(¹Patients were also seroreactive to *B. henselae* and/or *B. vinsonii subsp.berkhoffii*.)

1.4. Reservoirs

1.4.1 Cats

Bartonella sp.-related diseases in cats that also affect humans includes cat scratch disease, caused by *B. henselae* as well as other potentially fatal disorders affecting mostly immunocompromised people (Grazia et al., 2013). Cats can also be reservoir host for *B. clarridgeiae* (that can also cause cat scratch disease) and *B. koehlerae*, a causative agent of endocarditis in humans (Chomel et al., 2004). Two other species, *B. bovis* and *B. quintana* have been isolated from cat blood, but the mechanisms of transmission and the reservoir potential of these species in cats, have not been further studied (Chomel et al., 2004). In addition, *B. vinsonii subsp. berkhoffii* DNA was detected in the blood of a cat (Varanat et al., 2009) and cat myocardium tissue (Donovan et al., 2018).

Cats naturally infected with *Bartonella* spp. do not usually show clinical signs, however, cats coinfecting with both feline immunodeficiency virus and *B. henselae* show a higher incidence for gingivitis and lymphadenopathy (Ueno et al., 1996) and suffer from relapsing bacteremia over extended periods of time (Kordick et al., 1995). Co-infections with more than one *Bartonella* spp. are common (Breitschwerdt et al., 2000). Transmission among cats occurs via fleas, predominantly, but can also occur via other arthropod vectors (Mosbacher et al., 2011). This type of transmission seems to be essential; as uninfected cats kept together with infected ones in an ectoparasite-free environment do not become infected with *Bartonella* spp. and no direct transmission of

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B. henselae from cat to cat has been documented (Mosbacher et al., 2011 and Chomel et al., 1996).

Given the long lasting association of *B. henselae*, *B. clarridgeiae* and domestic cats, there have been adaptations between host and bacteria to facilitate co-existence and minimize pathogenic effects on the mammalian host (Guptill, 2010). However, there is evidence that cats may also suffer from a persistent infection, especially when infected with species not specifically adapted to the cat as reservoir host (e.g. *Bartonella vinsonii subsp. berkhoffii*) which can result in more serious clinical symptoms, such as osteomyelitis (Breitschwerdt et al., 2000). Some studies indicated a correlation between seroreactivity and stomatitis, uveitis and kidney and urinary tract-diseases (Ueno et al., 1996; Glaus et al., 1997 and Sykes et al., 2010).

Bartonella spp. have a highest prevalence in areas where conditions are most favorable for arthropod vectors, such as fleas. Many studies have been carried out in Europe, and the antibody prevalence in cats ranged from 8 to 53% (**Table 5**) (Grazia et al., 2013). *Bartonella* spp. have also been isolated from cat blood in various locations throughout the world (e.g. from San Francisco/USA, North Carolina/USA, Hawaii/USA, Japan, Sydney, New Zealand, the Netherlands, France, Indonesia and Germany) (Regier et al., 2016).

Table 5. Antibody prevalence of *Bartonella* sp. infection in the feline populations sampled in European countries. (Adapted from Grazia et al., 2013).

Country	Number of cats	Prevalence (%)	Reference
The Netherlands	163 (stray)	52	Bergmans et al., 1997
Austria	96	33	Allerberger et al., 1995
Switzerland	728	8	Glaus et al., 1997
Germany	713	15	Haimerl et al., 1999
	245	37.1	Morgenthal et al., 2012
France	94	53	Heller et al., 1997
	179	41	Gurfield et al., 2001
Spain	680	23.8	Ayllon et al., 2012
Italy	540	38	Fabbi et al., 2004
	1300 (stray)	23.1	Brunetti et al., 2013
	197	45.7	Persichetti et al., 2018
Scotland	78	15.3	Bennett et al., 2011

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1.4.2. Dogs

Dogs represent an incidental host for *Bartonella* spp. and two species are known to cause clinically apparent infections: *B. vinsonii* subsp. *berkhoffii* and *B. henselae*. (Chomel et al., 2006).

Dogs are suspected to be the main reservoir hosts for *B. vinsonii* subsp. *berkhoffii* because of the seroreactivity of dogs for this bacteria, recorded worldwide, together with a prolonged bacteremia (Chomel et al., 2006). This species may cause immune-mediated hemolytic anemia, neutrophil polyarthritis, neutrophil or granulomatous meningoencephalitis and uveitis in dogs, as suggested by many serological surveys (Boulouis et al., 2005). In addition, this bacteria can also cause endocarditis in large breed dogs, with a predisposition for aortic valve involvement and fever of unknown origin that can even start several months before the developing of endocarditis (Breitschwerdt et al., 2000). Myocarditis can, as well, be developed (without a direct association with endocarditis) and may result in syncope, arrhythmias or sudden death (Breitschwerdt et al., 2000).

As referred, domestic dogs may be incidental hosts for *B. henselae* and seroprevalence increases in warmer regions. A 27% seroprevalence in sick dogs vs. a 10% in healthy dogs have been reported in the United States (Mosbacher et al., 2011). There are also evidence that dogs are able to transmit *B. henselae* to humans via bites (Chomel et al., 2006 and Mosbacher et al., 2011). *B. henselae* can cause peliosis hepatis in dogs, a rare vascular condition of the liver characterized by a proliferation of the sinusoidal hepatic capillaries that results in cystic blood-filled cavities distributed randomly throughout the liver (Biswas and Rolain, 2010). Kitchell et al. (2000) reported for the first time a molecular evidence of *B. henselae* infection in a dog with peliosis hepatis.

Other species of the genus *Bartonella* such as *B. clarridgeiae*, *B. washoensis* and *B. quintana* have been found in dogs (Boulouis et al., 2005 and Chomel et al., 2006). All these three species were isolated from dogs suffering from endocarditis (Boulouis et al., 2005 and Chomel et al., 2006).

Finally, it is important to point out that to date, all *Bartonella* spp. identified in sick dogs are known as pathogenic or potentially pathogenic infectious agents for humans

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suggesting that dogs might act as useful sentinel species and important comparative models for human infections (Chomel et al., 2004).

1.5. Bartonellosis in Europe

Infections have been reported around the world, but the majority of the reports are on the North American perspective (Álvarez-Fernández et al., 2018). Historically, *Bartonella* spp. infections in Europe led to concern in the population during the two major world wars with the so-called “trench fever” but since then, not many new cases have been documented (Maurin et al., 1996; Sangare et al., 2014). To date, clinic-pathological data from humans in Europe are very low, being more common in domestic cats and dogs (Regier et al., 2016). However, despite its low prevalence, bartonellosis may be considered an underdiagnosed infectious disease in cats and dogs altogether (Álvarez-Fernández et al., 2018). For that reason, further research is needed to be performed in Europe to assess the relevance of *Bartonella* sp. infection in the spectrum of feline and canine diseases (Álvarez-Fernández et al., 2018).

1.5.1. Epidemiology, prevalence and distribution in Europe

More than 50 feline and canine bartonellosis seroprevalence studies have been conducted in different European countries, however, culture or PCR confirmed cases have been rarely reported (Álvarez-Fernández et al., 2018).

Records demonstrate that *Bartonella* spp. seroprevalence rates are higher in cats in European Mediterranean countries, probably because temperature and humidity are favorable for flea and tick infestations (Chomel et al., 2006). Moreover, *Bartonella* sp. antibody prevalence in cats ranges from 0% in Norway to 71.4% in Spain (Bergh et al., 2002 and Solano-Gallego et al., 2006). Co-infection with various combinations of *B. clarridgeiae*, *B. henselae* and *B. koehlerae* often approach 50–75% in feral cat populations worldwide (Chomel et al., 2010). Usually, the differences in serological or bacteremic prevalences are related to three main factors: the different climatic conditions, whether the cat population consisted of pet or stray cats and whether acaricide products were used routinely (Chomel et al., 2006).

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B. henselae infection has been reported in cats, dogs and humans among all European countries (Pennisi et al., 2013). *B. clarridgeiae* serological and molecular prevalence rates in Europe, varies from 17 to 36%, while *B. quintana* seroprevalence rates range from 0 to 18%, but more studies need to be performed to have accurate data about the epidemiology of these two species (Pennisi et al., 2013). Finally, although *B. koehlerae* and *B. bovis* have not yet been documented to infect cats in Europe, *B. koehlerae* DNA has been amplified from cat fleas in France (Rolain et al., 2003).

1.5.2. Bartonellosis in Portugal

There are only a few studies in Portugal that have focused on *Bartonella* sp. infections on cats. The first one, was carried out by Childs et al. (1995), concerning to the prevalence of Rochalimaea species in cats (during the time *Bartonella* species taxonomy was still unchanged). From that study, 14.3% of cats were considered positive for infection with *B. quintana*. The second one was carried out by Alves et al. (2009), focusing on domestic, sheltered and stray cats and fleas, reporting 64.9% of seropositive cats for *B. henselae* and 67.7% positive fleas, respectively by using a polymerase chain reaction (PCR) assay targeting *gltA* gene. The most recent study was performed in southern Portugal by Maia et al. (2014): 649 cats (320 domestic and 329 stray) from veterinary medical centers and animal shelters were tested and *Bartonella* spp. infections were evaluated by PCR targeting the *ITS* gene in blood samples. From that pool sample, 19 cats (2.9%) were positive for *Bartonella* spp. and *B. henselae* and *B. clarridgeiae* were identified post sequencing.

In regards of dogs, one thousand and ten dogs from veterinary medical centers and animal shelters were screened for *Bartonella* sp. by a cPCR in southern Portugal by Maia et al. (2015), all of them tested negative. There is also a study on fleas from mice, rats and one hedgehog performed in Madeira Island and mainland Portugal by De Sousa et al. (2006), in which four *Ornithophaga* fleas were positive by PCR to new *Bartonella* sp. genotypes closely related to *Bartonella elizabethae*. All fleas came from mainland Portugal collections and not from Madeira.

For humans, cases have not been frequent and complete reports are difficult to find in scientific databases. Despite that, three cases for *Bartonella* sp. infection have

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been documented in recent years. The first one involves an immunocompetent kitten owner of 44 years, who, presented a papule in a finger of the right hand and regional lymphadenopathies, whose diagnosis was performed by serology and PCR on lymph node biopsy (Murinello et al., 2010). The second case involved an adolescent, who was in contact with a cat and presented right pre-auricular adenopathy; *B. henselae* was detected by serology (Dias et al., 2012). Lastly, the third case corresponded to a healthy 29-year-old man with lymphadenopathy in the axillary and *Bartonella* spp. was detected after blood and cutaneous lesions biopsy testing (Costa et al., 2011).

In this context, more studies need to be performed in order to determine the current prevalence of *Bartonella* spp. in Portugal for cats (the main reservoirs), and dogs, also including humans and potential vectors, such as ticks. Therefore, the present investigation aimed to survey *Bartonella* sp. DNA extracted from cats, dogs, and humans from Portugal, using a qPCR assay targeting *nuoG* gene. Moreover, a characterization of the *Bartonella* sp. detected was also carried out using *gltA* and *ribC* based cPCR in order to identify the *Bartonella* spp. circulating in Lisbon, Portugal.

2. Materials and methods

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2.1. Sample collection

2.1.1. Ticks

The majority of the ticks used in this work were obtained under a previously developed research project. Tick collections were performed from 2012 to 2018 covering 19 geographical points of mainland Portugal. Ticks were collected by dragging-flagging vegetation, as described by Aeschlimann (1972), examined under a stereo-microscope, and morphologically identified using the taxonomic keys previously described by Santos-Silva et al. (2011) and Walker et al. (2000). Each specimen was placed in a 1.5mL labeled microtube and stored in 70% ethanol at -20°C, until DNA extraction.

2.1.2. Domestic animals

Non-domiciled cats and dogs participating in the sterilization program promoted by Casa dos Animais de Lisboa (CAL) were sampled from January to July 2019. Blood samples were collected for other clinical applications (e.g., pathological or biochemistry analysis), and a small amount corresponding to 400 µl of feline and 400 µl of canine EDTA whole blood, divided into two aliquots, were gently donated to this study. Blood was collected by trained and authorized veterinarians, from the cephalic or jugular vein of the animals immediately before the surgical procedure, under anesthesia. Samples were classified according to their localities of origin, transported on ice and further stored at -80°C until use.

2.1.3. Humans

Healthy volunteers frequently exposed to bites or cat scratches like veterinarians, veterinary technicians, participants of associations for the protection of homeless animals and cat owners were recruited for this study, from February to July 2019. After reading and signing an informed written consent, approximately 1 mL of EDTA whole blood was collected from the volunteers' median cubital vein by an experienced and authorized biomedical researcher; samples were divided into two aliquots, transported on ice and stored at -80°C until use.

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2.2. Ethics approval

The Committee on the Ethics of Animal Experiments from the Institute of Hygiene and Tropical Medicine (CEIHMT) approved this study under the permit 01.19 (**Annex 1**), as well as the informed consent (**Annex 2**) used to volunteer participation of humans in this research. The principle of the three R's, to replace, reduce and refine the use of animals for scientific purpose was followed.

2.3. Objectives and experimental design

Tick samples and blood from cats, dogs and humans were used for DNA extraction; a conventional PCR (cPCR) was used to confirm the integrity of the DNA (18 rRNA was amplified for ticks and *GAPDH* for the rest of the samples). Because cPCR assays have limited sensitivity for *Bartonella* sp. detection when compared with qPCR (André et al., 2016), a quantitative real time PCR for *nuoG* gene was performed to screen *Bartonella* spp. in all samples as the first step for molecular detection. In addition, endogenous *Bartonella* sp. genes *gltA* and *ribC* cPCR assays have already been used successfully for *Bartonella* spp. characterization in prior studies (Braga et al., 2012 and Alves et al., 2009); so as a second step, two following cPCR for *gltA* and *ribC* genes were performed for molecular characterization and species identification for all *nuoG* positive samples. Finally, blood samples from dogs and humans were cultured in liquid medium and subcultured in solid medium. DNA extracted from both cultures were also tested as previously described. The flowchart of the culture and PCR-based procedures is presented in **Figure 2**.

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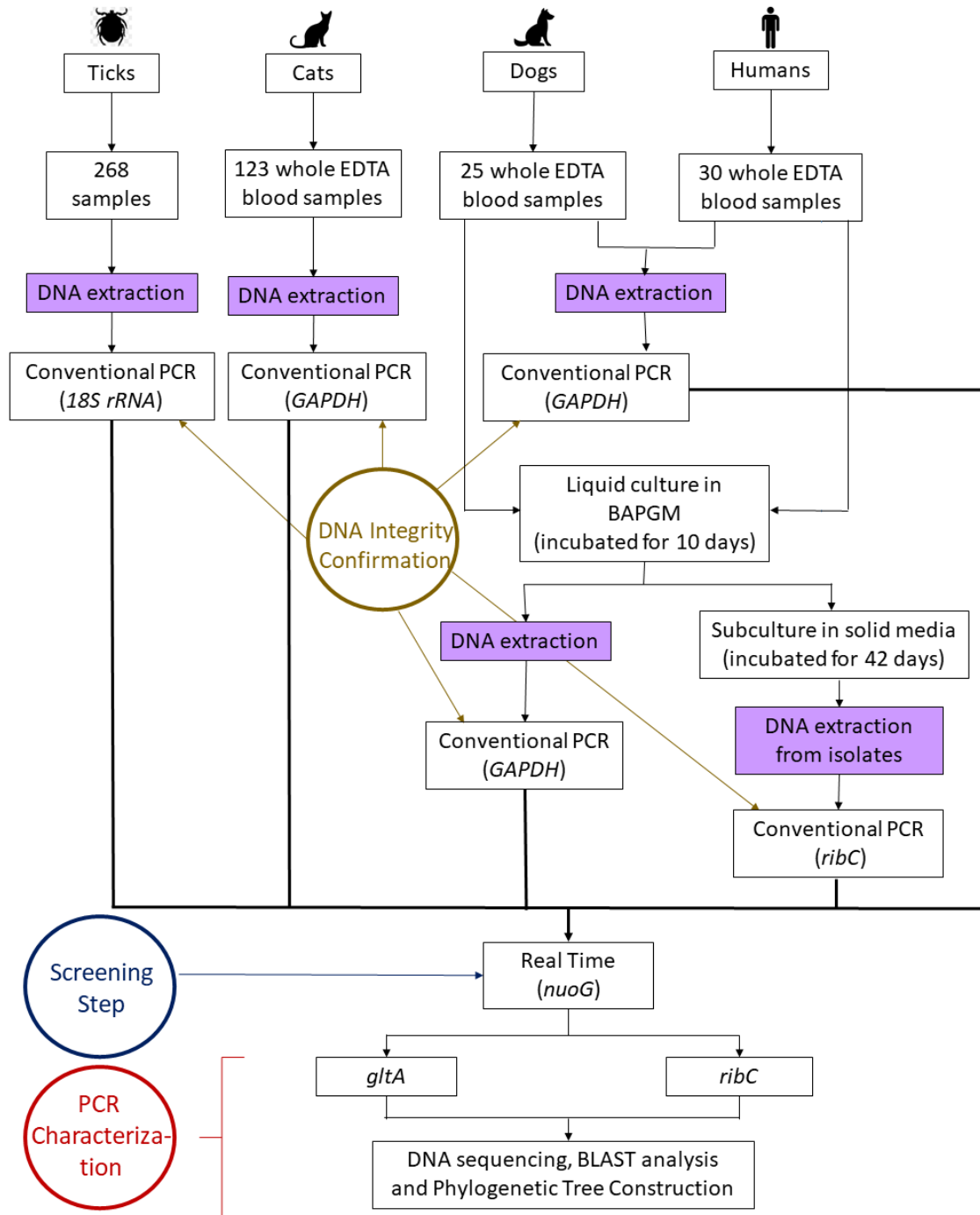


Figure 2. Flowchart of the culture and PCR-based procedures performed to determine *Bartonella* sp. prevalence in ticks and blood samples from cats, dogs and humans.

2. Materials and methods

2.4. DNA extraction

DNA from ticks was extracted by using alkaline hydrolysis, as previously described (Alekseev et al., 2001). Briefly, ticks were taken from the 70% ethanol solution, gently dried, and boiled for 20 minutes in 100 μ L of 0.7 M ammonium hydroxide to free the DNA. After being cooled, the microtube with the lysate was opened and incubated for 20 minutes at 90°C to evaporate the ammonia. DNA from cats, dogs and humans blood was extracted by using the NZY Blood gDNA Isolation Kit (Nzytech, Lisbon, Portugal), and following the manufacturer's protocol. Purified DNA samples were eluted in 50 μ L.

The DNA concentration was estimated by using ND-1000 Nanodrop Spectrophotometer (Nanodrop, Thermo Scientific, USA), along with determining absorbance ratio at 260/280 and 260/230 nm for evaluating the quality of obtained DNA, and then samples were stored at -20°C until use.

2.5. Polymerase chain reaction (PCR)

2.5.1. cPCR for DNA integrity confirmation

In order to confirm the DNA integrity and the absence of PCR inhibitors, eliminating the possibility of false negative results, a conventional PCR targeting mammalian and ticks endogenous genes was performed.

The gene coding for the mammalian glyceraldehyde-3-phosphate dehydrogenase protein family (*GAPDH*) was used as endogenous gene for cats, dogs and humans' blood and cell culture. A cPCR that amplifies a fragment of 437 bp was performed using the primers GAPDH-F: CCTTCATTGACCTCAACTACAT, and GAPDH-R: CCAAAGTTGTCATGGATGACC, previously described (Birkenheuer et al., 2003). DNA amplification was carried out in 25 μ l reaction volumes containing 12.5 μ l of NZY Taq II 2x Green Master Mix (Nzytech) containing NZY Taq II DNA polymerase, dNTPs, MgCl₂ and loading dye, 1 μ M of each primer, 1 μ l of DNA template and nuclease-free water up to the final volume. Negative control were prepared with no template. The reaction mixture was subjected to thermal profile consisting of an initial denaturation at 94°C for 3 minutes, 35 cycles consisting of denaturation at 94°C for 30 seconds, annealing

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at 50°C for 30 seconds and extension at 72°C for 1 minute, followed by final extension at 72°C for 5 minutes. All steps of conventional PCR were performed in a T100™ Thermal Cycler (BioRad™, CA, USA).

The gene coding for the small subunit ribosomal RNA (18S rRNA) of arthropods was used as endogenous gene for ticks. A cPCR that amplifies a fragment of 653 bp was performed using the primers 18S-F: AACCTGGTTGATCCTGCCA, and 18S-R: CTGAGATCCAACTACGAGCTT, previously described (Mangold et al., 1998). The reaction mixture was prepared as described above, and subjected to thermal profile consisting of an initial denaturation at 95°C for 2 minutes, 35 cycles consisting of denaturation at 92°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 45 seconds, followed by final extension at 72°C for 5 minutes.

Five microliters of each amplified product in the conventional PCR assays were subjected to horizontal electrophoresis in 1.5% agarose gel stained with 0.03 µL / mL of Green Safe Premiun (Nzytech,) in 0.5x TEB run buffer (20 mM Tris, 20 mM boric acid, 0.5 mM EDTA, pH 7.2). Electrophoresis was performed at 100 V / 400 W for 40 minutes. Nzytech DNA Ladder VIII molecular weight marker (Nzytech) was used to confirm the approximate size of the amplified products. The electrophoresis gel were imaged under ultraviolet light transilluminator (GVM20X-E Transilluminator, Syngene)

2.5.2. Molecular detection of *Bartonella* sp.

All positive samples for the endogenous genes were subjected to a quantitative PCR (qPCR) targeting a fragment of the NADH ubiquinone oxireductase subunit G (*nuoG*) gene, using the primers F-Bart: 5'-CAATCTTCTTTTGCTTCACC-3', R-Bart: 5'-TCAGGGCTTTATGTGAATAC-3', and the hydrolysis probe FAM-5'-TTYGTCATTTGAACACG-3'[BHQ1]-3' (André et al., 2015). The qPCR assays were performed for a final volume of 10µl, containing 1µ of DNA template, 0.6 µM of each primer and probe, 5 µl of Xpert Fast PROBE 2X Master Mix and ultra-pure sterilized water (Thermo Scientific) *q.s.p.* 10 µL. The amplification conditions were 95°C for 3 minutes followed by 40 cycles at 95°C for 10 seconds and 52.8°C for 30 seconds (André et al., 2015). qPCR amplifications were conducted in low-profile multi-plate unskirted PCR plates (BioRad™), using a CFX96 Thermal Cycler (BioRad™, CA, USA). Standard

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curves were constructed with serial dilutions of plasmid DNA (pIDTSMART—Integrated DNA Technologies) (1.0×10^7 to 1.0×10^9 copies/ μL), which encodes an 83 bp *B. henselae-nuoG* gene fragment. The number of plasmid copies was determined by using the following formula: $(X \text{g}/\mu\text{L DNA} / [\text{plasmid length in bp} \times 660]) \times 6.022 \times 10^{23} \times \text{plasmid copies}/\mu\text{L}$.

2.5.3. Molecular characterization of *Bartonella* spp.

All positive samples for the qPCR *nuoG* gene were selected to go under cPCR assays based on 750 bp fragment of the citrate synthase (*gltA*) gene (Billeter et al., 2011), and 585 bp fragment of riboflavin synthase (*ribC*) (Johnson et al., 2003) to perform the molecular characterization for the *Bartonella* spp. detected. REPLI-g UltraFast Mini Kit (QIAGEN, Hilden, Germany) was used to replicate one of the qPCR positive sample (blood of the cat 79) in order to use it as positive control. Ultra-pure sterilized water (Thermo Scientific) was used as negative control. The protocol used for each gene is described below.

2.5.3.1. cPCR for the *gltA* gene of *Bartonella* spp.

The fragment of the *gltA* gene was amplified with the primers 443F: 5'-GCTATFTCTGCATTCTATCA-3' and 1210_R: 5'-GATCYTCAATCATTCTTTCCA-3' (Billeter et al., 2011), in 25 μL reaction volumes containing 12.5 μL of NZYTaQ II 2x Green Master Mix (Nzytech.), 1 μM of each primer, 1 μL of DNA template and nuclease-free water q.s.p. 10 μL . The reaction mixture was subjected to thermal profile consisting of an initial denaturation at 95°C for 5 minutes, 35 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds and extension at 72°C for 1 minute, followed by final extension at 72°C for 5 minutes.

2.5.3.2. cPCR for the *ribC* gene of *Bartonella* spp.

The fragment of the *ribC* gene was amplified with the primers BARTON-1: 5'-TAACCGATATTGGTTGTGTTGAAG-3' and BARTON-2: 5'-TAAAGCTAGAAAGTCTGG CAACATAACG-3' (Johnson et al., 2003), in 25 μL reaction volumes containing 12.5 μL of NZYTaQ II 2x Green Master Mix (Nzytech.), 1

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μM of each primer, 1 μl of DNA template and nuclease-free water q.s.p. 10 μL. The reaction mixture was subjected to thermal profile consisting of an initial denaturation at 95°C for 5 minutes, 35 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 1 minute, followed by final extension at 72°C for 5 minutes.

2.5.3.3. Molecular cloning for detection of coinfection

In order to detect a coinfection with two or more species of *Bartonella* sp., a molecular cloning was performed, when applicable, using the kit InsTAclone PCR Cloning Kit (Fermentas, Baden-Wurttemberg, Germany), a system for direct one-step cloning of cPCR products with 3'dA overhangs that uses the plasmid pTZ57R/T. For the cloning, a conventional PCR for the *ribC* gene was performed, following the conditions previously described on 2.5.3.2. The resulting amplicon was purified using the NZYGelpure kit (NZYtech), following the manufacturer's recommendations. The InsTAclone PCR Cloning Kit manufacturer's protocol was followed for the molecular cloning to have place, using 50 μl of competent *E. coli* cells (NZYtech).

Plates were done using LB Agar (NZYtech). Fifty microliters of transformed bacteria were plated and incubated at 37°C, overnight. To detect if the ligation or transformation was successful, white colonies were tested by a conventional PCR for *ribC* gene, following the conditions described on 2.5.3.2. Positive colonies were subcultured on 3 ml of LB Medium (Liofilchem, Teramo, Italy) at 37°C and under 200 rpm shaking overnight. Finally, a purification was carried out using the NZY Miniprep kit (NZYtech) following the manufacturer recommendations, and 10 μl of purified samples together with 3 μl of *M3* forward primer was sent to StabVida (Lisbon, Portugal), for sequencing.

2.6. Amplified product purification and sequencing

Positive amplicons, for both *gltA* and *ribC* cPCR were purified using the NZYGelpure kit (NZYtech, Lisbon, Portugal), following the manufacturer's recommendation and sent for Sanger sequencing at StabVida.

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2.7. Sequence analysis

All electropherograms were analyzed using Bioedit Sequence Alignment Editor (Hall, 1999), in order to increase the quality level of the sequences, based on each nucleotide peak, knowing all had to surpass the minimum quality level of 20 per peak. After that, the sequences were manually trimmed, and submitted to the Basic Local Alignment Search Tool (BLAST) (Altschull et al., 1990) for identification by comparison with sequences available in the GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>).

Sequences were grouped in four datasets (*B. henselae gltA*, *B. clarridgeiae gltA*, *B. henselae ribC* and *B. clarridgeiae ribC*), and a ClustalW multiple alignment was performed in Bioedit for each group of gene sequences. Tests were conducted using the software DNA Sequence Polymorphism (DnaSP) v.6.12.03 (Rozas et al., 1995) to determine the number of haplotypes (h) and to measure the genetic differentiation (i.e., haplotype diversity; Hd) between sequences for the datasets. The haplotypes were generated based on nucleotide sequences with gaps or missing data and excluding the invariable sites. MEGA v.6.06 software (Tamura et al., 2013) was used to calculate the amount of nucleotide variation among the sequences of the generated haplotypes by making pairwise comparisons using the p-distance model with 1,000 bootstrap replications.

In regards of the phylogenetic tree construction, a ClustalW multiple alignment was performed in Bioedit for each group of gene sequences (*gltA* and *ribC*), including a representative sequences of each haplotype obtained in this study, together with other sequences selected from the GenBank. Likelihood-mapping analyses were performed for both datasets after alignment, to estimate the support of a hypothesized internal branch and visualize the phylogenetic content of the dataset before computing an overall tree, using the TREE-PUZZLE v5.3 program (Schmidt et al., 2002). Only the datasets with $\geq 90\%$ resolved quartets were used for tree reconstruction. The MEGA v.6.06 software was used to obtain the best model to describe the substitution pattern. A maximum-likelihood (ML) tree was constructed based on a bootstrapping method with 1,000 replicates and a Kimura 2-parameter model. Finally, tree edition was performed using FigTree v.1.4.4 (Rambaut, 2006).

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2.8. Blood culture

2.8.1. Medium preparation and culture pre-treatment

For 450 mL of liquid medium BAPGM was necessary to mixture 0.05 mg β -Nicotinamide Adenine Dinucleotideo Hydrate (Sigma Aldrich), 0.625 mg β -Nicotinamide Adenine Dinucleotideo Phosphate Reduced Tetrasodium Salt Hydrate (Alfa Aesar, Kandel, Germany), 1 mg Adenosine 5-Triphosphato Disodium Salt (Sigma Aldrich), 1 mg Sodium Pyruvate (Sigma Aldrich) and 1 g Yeast Extract (Sigma Aldrich) on 450 ml of medium IPL-41 Insect Medium (Sigma Aldrich) (Gutierrez et al., 2017). The homogenized solution was filtered under a filter system of 0.22 μ m. and supplemented with 5% amphotericin to reduce fungal contamination (Kosoy et al. 1997). For each tube, 2mL of medium and was used 200 μ l of blood sample was added.

In regards of the solid culture, chocolate agar supplemented plates were used (VWR Chemicals, Leuven, Belgium). Furthermore, to reduce the impact of coinfecting bacteria for direct cultures, 100 μ L of the blood samples were diluted and homogenized in 100 μ L liquid medium (1:1) (Kosoy et al. 1997). For sub-cultures, 200 μ l of the previously incubated liquid cultures were used.

Samples were kept frozen at -80°C and thaw only before being plated, in order to promote the release of intraerythrocytic *Bartonella* (Welch et al. 1992). For the liquid culture, incubation was performed at 37°C and 5% of CO₂ under constant shaking for 10 days. Chocolate-agar plates cultures were incubated at 37°C and 5% of CO₂ for 42 days in a Sanyo CO² Incubator (model MCO-20AIC).

2.8.2. Cultures DNA extraction

For liquid cultures, 2 mL of cultured blood (after 10 days of incubation) was centrifuged for 20 minutes at 15000 rpm. The supernatant was discarded and used for extraction using QIAGEN Genomic DNA Extraction kit (QIAGEN) following the manufacturer's recommendation. Purified DNA samples were eluted in 30 μ L.

In regards of solid cultures, colonies were selected for extraction if presenting at least one of the following characteristics: slow growing (at least no visible colony during

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the first week) or small size, round shape and segregated from one another (Ventura and Padilla, 2006).

Selected colonies were taken from the plates and diluted in 1 mL of sterilized water for each sample before centrifugation at 15000 rpm for 20 minutes. After the supernatant was discarded, extraction was performed by using the NZY Blood gDNA Isolation Kit (Nzytech) following the manufacturer's protocol. Purified DNA samples were eluted in 30 μ L.

3. Results

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3.1 Ticks

3.1.1. Sample Collection

A total of 268 specimens were collected in 19 geographical points from 8 districts of mainland Portugal from 2012 to 2018 (**Figure 3**). Of these, 114 (42.53%) were identified as *Ixodes ricinus*, 18 (6.71%) as *Ixodes* sp. and 136 (50.75%) as *Rhipicephalus sanguineus* sensu lato. For *I. ricinus*, 38 (33.3%) specimens were female, 56 (49.12%) were male and 20 (17.54%) nymph; in addition, all the 18 (100%) individuals classified as *Ixodes* sp. were nymphs. In regards to *R. sanguineus*, 68 (50%) individuals were females and 68 (50%) males.

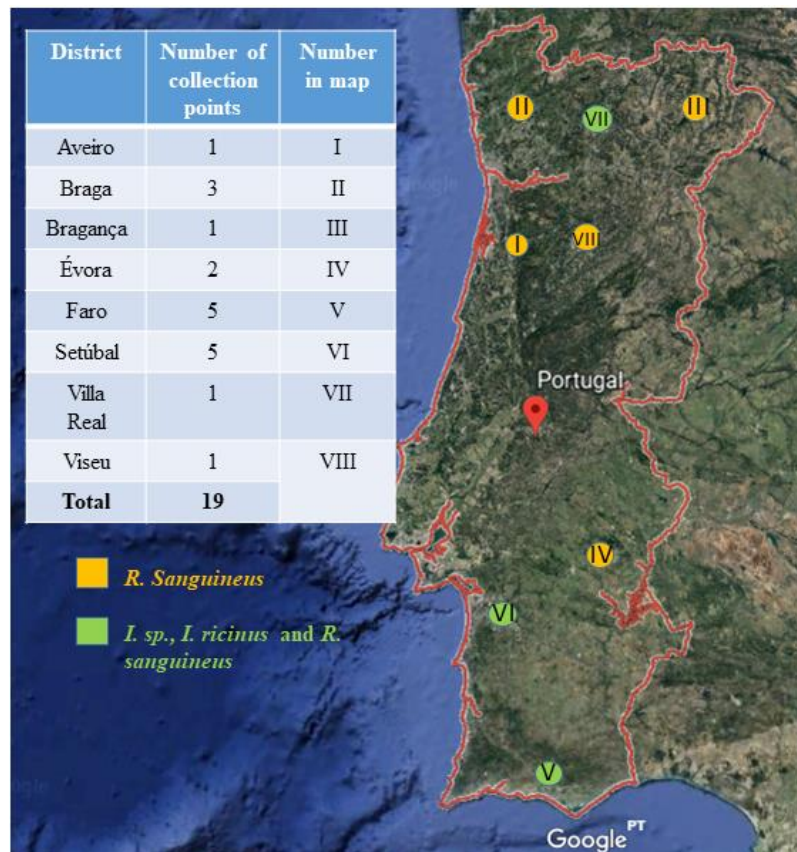


Figure 3. Tick collected in Portugal mainland classified by district, with information of the number of collection points per district and whether *R. sanguineus* or both *R. sanguineus* and *Ixodes* spp. were identified. Image adapted from Google Earth v.7.3.2.5491.

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3.1.2. DNA integrity confirmation

After DNA extraction using alkaline hydrolysis (Alekseev et al., 2001), all 268 samples tested positive for the endogenous gene 18S rRNA (Mangold et al., 1998) confirming the integrity of the DNA (**Figure 4**).

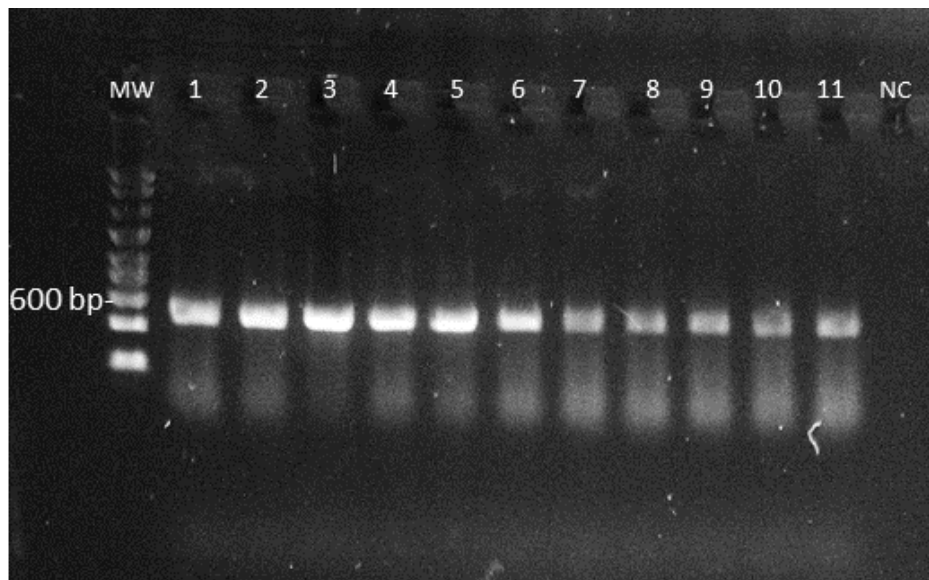


Figure 4. Electrophoresis in 1.5% agarose gel stained with 0.03 μL / mL of Green Safe Premium (Nzytech) showing the amplicons for a fragment of 653 bp of the 18S rRNA gene. (MW: molecular weight size marker; wells 1 to 11: positive amplicons for samples 12 to 22; NC: negative control).

3.1.3. Molecular detection for *Bartonella* sp.

All tick samples were negative for the qPCR targeting the *nuoG* *Bartonella* sp. endogenous gene. The mean efficiency (E) of the qPCR reactions for the *nuoG* gene was $101.98\% \pm 6.96$ (92.9% to 112.8%), and the slope ranged from -3.463 ($R^2=0.999$) to -3.285 ($R^2=0.994$). -3.285. Consequently, molecular characterization was not performed.

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3.2. Cats

3.2.1. Sample collection

From 123 samples coming from 37 different geographical areas in Lisbon and distributed in 22 Lisbon localities, 112 (91.05%) corresponded to adult cats and 11 (8.87%) to juveniles. Also, 73 (58.87%) were female and 51 (45.16%) were male. Furthermore, most of them (95.16%) were European Shorthair cats (most common cat breed in Europe) and only 9 (7.25%) were flea infested (**Annex 3**).

3.2.2. DNA integrity confirmation

All 123 extracted DNA samples were quantified by Nanodrop Spectrophotometer (Nanodrop), presenting concentrations ranged from 3 ng/ μ L to 154.8 ng/ μ L. All were positive for a cPCR targeting a fragment of the mammalian gene *GAPDH*, confirming the integrity of DNA (**Figure 5**).

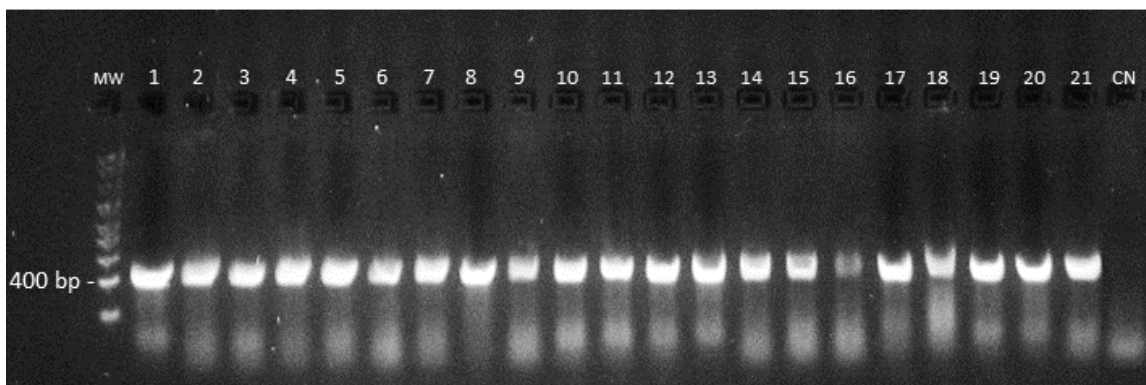


Figure 5. Electrophoresis in 1.5% agarose gel stained with 0.03 μ L / mL of Green Safe Premium (Nzytech) showing cat DNA amplicons for a fragment of 437 bp of the *GAPDH* gene. (MW: molecular weight size marker; MW: wells; 1-24: amplicons for samples 25 to 35, respectively; NC: negative control).

3.2.3. Molecular detection for *Bartonella* sp.

A qPCR targeting an 83 bp fragment of the *nuoG* gene was performed for all 123 samples to screen and quantify *Bartonella* sp. DNA. Of these, 25 (20.32%) samples were

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positive, corresponding to samples number 2, 16, 19, 22, 27, 28, 29, 33, 37, 46, 52, 57, 62, 71, 79, 80, 86, 90, 95, 97, 101, 107, 108, 118, and 109. Information regarding breed, sex, age, local of origin and presence of flea infestation are found in **Annex 3**.

Of these infected cats, 13 were females, corresponding to the 17.1% of all female cats, and 12 were males, representing the 23.52% of all males. In regards to age, 5 of the 11 juvenile cats were infected (45.45%), as well as 20 (17.85%) of the total of adults. In addition, only three from nine (33.33%) presented flea infestation, being all females. Furthermore, 28% of the infected cats were found in Alvalade, 8% in Misericórdia and 8% in Campolide.

The mean efficiency (E) of the qPCR reactions for the *nuoG* gene, R^2 , slope and y-interceptions ranged from 92.40% to 111.90% (average of 94.78%), 0.972 to 1 (average of 0.994), -3.521 to -3.320 (average of -3.449) and 1.974 to 42.265 (average of 29.139), respectively. Quantification ranged from 2.78×10 to 1.03×10^5 copies/ μ L. (**Table 6**).

Table 6. Positive cat blood samples for *nuoG* qPCR assay with the correspondent reaction parameters.

Positive Samples	Quantification (copies/ μ l)	Efficiency	R^2	Slope	y-interception
2	9.53E+01	100.10%	0.972	-3.320	3.029
16	4.07E+02	95.40%	0.996	-3.437	41.439
19	2.62E+02	92.40%	0.997	-3.520	1.974
22	1.35E+03	111.90%	0.997	-2.827	6.768
27	3.23E+03	92.40%	0.997	-3.520	1.974
28	9.47E+04	92.40%	0.997	-3.520	1.974
29	6.29E+02	92.40%	0.997	-3.520	1.974
33	1.70E+02	92.40%	0.997	-3.520	1.974
37	1.14E+04	92.40%	0.997	-3.520	1.974
46	4.39E+03	92.30%	0.993	-3.521	41.293
52	2.19E+03	92.30%	0.993	-3.521	41.293
57	7.70E+02	93.50%	0.978	-3.489	41.418
62	3.17E+03	93.50%	0.978	-3.489	41.418
71	1.62E+02	93.50%	0.978	-3.489	41.418
79	1.03E+05	92.80%	1	-3.508	42.265
80	2.82E+03	92.80%	1	-3.508	42.265
86	6.15E+02	92.80%	1	-3.508	42.265
90	1.57E+02	92.80%	1	-3.508	42.265
95	2.78E+01	98.50%	0.999	-3.358	40.737

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Table 6 (continued).

97	6.37E+02	92.80%	1	-3.508	42.265
101	3.24E+02	98.50%	0.999	-3.358	40.737
107	2.03E+02	95.40%	0.996	-3.437	41.439
108	2.48E+02	95.40%	0.996	-3.437	41.439
118	2.12E+02	95.40%	0.996	-3.437	41.439
119	1.41E+02	95.40%	0.996	-3.437	41.439

3.2.4. Molecular characterization of *Bartonella* spp.

3.2.4.1. cPCR for the *gltA* gene of *Bartonella* spp.

Molecular characterization of positive samples was made using a fragment of 750 bp for the *gltA* gene amplified by conventional PCR. All 25 positive samples were also positive for *gltA* (**Figure 6**).

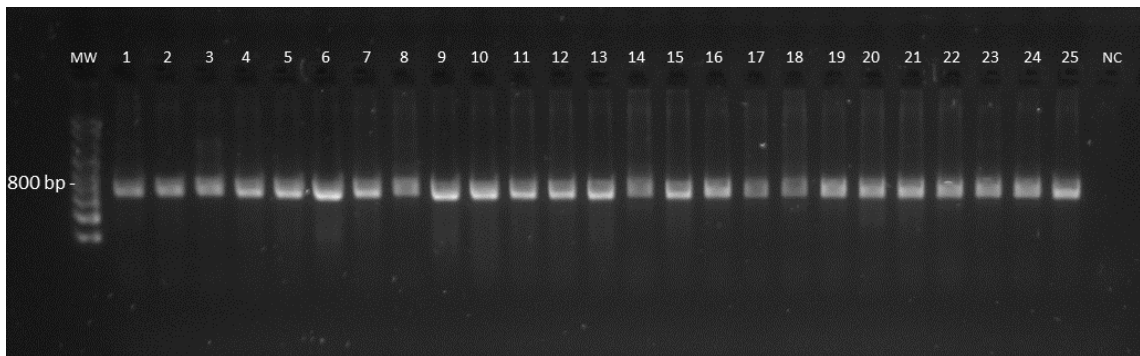


Figure 6. Electrophoresis in 1.5% agarose gel stained with 0.03 μL / mL of Green Safe Premium (Nzytech) showing the amplicons for a fragment of 750 bp of the *gltA* gene from previously positive samples for *nuoG* qPCR. (MW: molecular weight size marker; wells 1-25 correspond to the following samples: 2, 16, 19, 22, 27, 28, 29, 33, 37, 46, 52, 57, 62, 71, 79, 80, 86, 90, 95, 97, 101, 107, 108, 118, and 109, respectively; NC: negative control).

Regarding the BLAST analysis for all the 25 sequences amplifying for the *gltA* gene, 13 (52%) presented a level of identity with *B. henselae* ranging from 99% to 100% and 11 (44%) an identity ranging from 99% to 100% with *B. clarridgeiae* (**Table 7**).

From the group of sequences characterized as *B. henselae*, eight (61.53%) had 99% of identity with *B. henselae* strain Houston-I (GenBank accession number CP020742) and five (38.46%) an identity ranging from 99% to 100% with *B. henselae*

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isolated from cat fleas from Chile (GenBank accession number KY913625). From the group characterized as *B. clarridgeiae*, eight (72.72%) presented an identity of 99% with *B. clarridgeiae* strain 73 (Genbank accession number FN645454), two (18.18%) showed a 100% identity with *B. clarridgeiae* isolated from cat fleas from Chile (GenBank accession number KY913629), and one (9.09%) a 100% identity with a second *B. clarridgeiae* also isolated from cat fleas from Chile (GenBank accession number KY913636). One sequence (corresponding to sample 108) was not analyzed due to the presence of two overlapping sequences and even though a molecular cloning was tried, the resulting sequences were undetermined, however, a coinfection of both *B. henselae* and *B. clarridgeiae* was suspected.

Table 7. BLAST identity percentages for *gltA* amplicons from cat blood collected in Lisbon, Portugal.

Sample	GenBank accession number	E value	Identity (%)	BLAST Query	Maximum Identity
2	CP020742	0.0	99	<i>B. henselae</i> strain Houston-I	99
16	CP020742	0.0	99	<i>B. henselae</i> strain Houston-I chromosome, complete genome	99
19	KY913636	0.0	100	<i>B. clarridgeiae</i> clone 147_14 citrate synthase (<i>gltA</i>) gene, partial cds	100
22	FN645454	0.0	99	<i>B. clarridgeiae</i> strain 73, complete genome	99
27	CP020742	0.0	99	<i>B. henselae</i> strain Houston-I chromosome, complete genome	99
28	FN645454	0.0	99	<i>B. clarridgeiae</i> strain 73, complete genome	99
29	CP020742	0.0	99	<i>B. henselae</i> strain Houston-I chromosome, complete genome	99
33	FN645454	0.0	99	<i>B. clarridgeiae</i> strain 73, complete genome	99
37	KY913625	0.0	99	<i>B. henselae</i> clone Gris citrate synthase (<i>gltA</i>) gene, partial cds	99
46	FN645454	0.0	99	<i>B. clarridgeiae</i> strain 73, complete genome	99

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Table 7 (continued).

52	FN645454	0.0	99	<i>B. clarridgeiae</i> strain 73, complete genome
57	CP020742	0.0	99	<i>B. henselae</i> strain Houston-I chromosome, complete genome
62	CP020742	0.0	99	<i>B. henselae</i> strain Houston-I chromosome, complete genome
71	FN645454	0.0	99	<i>B. clarridgeiae</i> strain 73, complete genome
79	FN645454	0.0	99	<i>B. clarridgeiae</i> strain 73, complete genome
80	FN645454	0.0	99	<i>B. clarridgeiae</i> strain 73, complete genome
86	KY913625	0.0	100	<i>B. henselae</i> clone Gris citrate synthase (<i>gltA</i>) gene, partial cds
90	KY913625	0.0	100	<i>B. henselae</i> clone Gris citrate synthase (<i>gltA</i>) gene, partial cds
95	CP020742	0.0	99	<i>B. henselae</i> strain Houston-I chromosome, complete genome
97	KY913629	0.0	100	<i>B. clarridgeiae</i> clone 774 citrate synthase (<i>gltA</i>) gene, partial cds
101	CP020742	0.0	99	<i>B. henselae</i> strain Houston-I chromosome, complete genome
107	KY913625	0.0	100	<i>B. henselae</i> clone Gris citrate synthase (<i>gltA</i>) gene, partial cds
118	KY913629	0.0	100	<i>B. clarridgeiae</i> clone 774 citrate synthase (<i>gltA</i>) gene, partial cds
119	KY913625	0.0	100	<i>B. henselae</i> clone Gris citrate synthase (<i>gltA</i>) gene, partial cds

3.2.4.2. cPCR for the *ribC* gene of *Bartonella* spp.

A conventional PCR targeting a fragment of 585 bp for the *ribC* gene tested all 25 positive samples for *nuoG* to perform a molecular characterization. All samples were amplified for the target gene, except the sample 71 (**Figure 7**).

3. Results

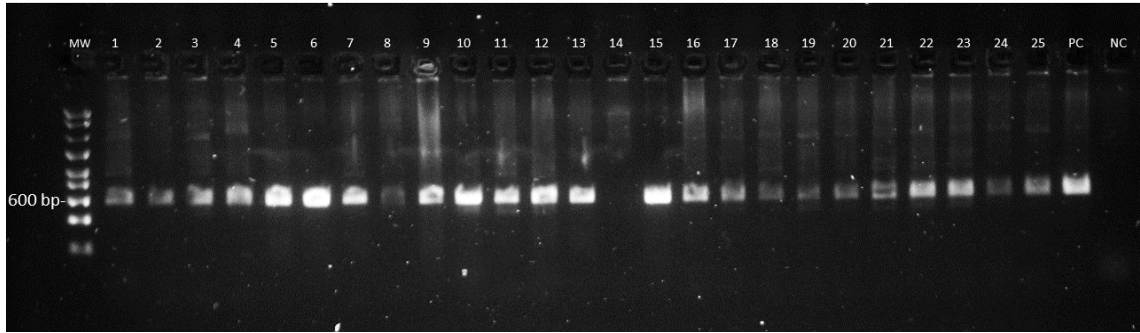


Figure 7. Electrophoresis in 1.5% agarose gel stained with 0.03 μL / mL of Green Safe Premium (Nzytech) showing the amplicons for *ribC* gene (585 bp) from previously positive samples for *nuoG* qPCR. (MW: molecular weight size marker; wells 1-25 correspond to the following samples: 2, 16, 19, 22, 27, 28, 29, 33, 37, 46, 52, 57, 62, 71, 79, 80, 86, 90, 95, 97, 101, 107, 108, 118, and 109, respectively; NC: negative control).

In regards of the BLAST analysis for all the 24 *ribC* sequences, 13 (54.16%) presented an identity with *B. henselae* ranging from 99.61% to 100%, 10 (41.66%) an identity ranging from 99.81% to 100% with *B. clarridgeiae* and 1 (4.16%) a coinfection of both *B. henselae* and *B. clarridgeiae* determined by molecular cloning (see 3.2.5.). From the group of sequences characterized as *B. henselae*, eight (61.53%) had a range of 99.61% to 99.81% of identity with *B. henselae* isolated from mammal fleas in South Carolina, U.S.A. (GenBank accession number AY953284), five (38.46%) an identity ranging from 92.62% to 100% with *B. henselae* strain Houston-I (GenBank accession number CP020742) and one (7.69%) showed 100% of identity with a *B. clarridgeiae* isolated from cats in Brazil (GenBank accession number HM588661). Moreover, from the group characterized as *B. clarridgeiae*, all 11 sequences (100%) presented an identity ranging from 99.81% to 100% with *B. clarridgeiae* proceeding from cats in southern Brazil (Genbank accession number KR092386) (**Table 8**).

Table 8. BLAST identity percentages for *ribC* amplicons from cat blood collected in Lisbon, Portugal.

Sample	GenBank accession number	E value	Identity (%)	BLAST Maximum Identity Query
2	AY953284	0.0	99.62	<i>B. henselae ribC</i> gene.

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Table 8 (continued).

16	CP020742	0.0	100	<i>B. henselae</i> strain Houston-I chromosome, complete genome
19	KR092386	0.0	99.81	<i>B. clarridgeiae</i> strain VG <i>ribC</i> gene, partial cds
22	KR092386	0.0	100	<i>B. clarridgeiae</i> strain VG <i>ribC</i> gene, partial cds
27	AY953284	0.0	99.61	<i>B. henselae</i> riboflavin synthase (<i>ribC</i>) gene, partial sequence
28	KR092386	0.0	100	<i>B. clarridgeiae</i> strain VG <i>ribC</i> gene, partial cds
29	AY953284	0.0	99.80	<i>B. henselae</i> riboflavin synthase (<i>ribC</i>) gene, partial sequence
33	KR092386	0.0	100	<i>B. clarridgeiae</i> strain VG <i>ribC</i> gene, partial cds
37	AY953284	0.0	99.81	<i>B. henselae</i> riboflavin synthase (<i>ribC</i>) gene, partial sequence
46	KR092386	0.0	100	<i>B. clarridgeiae</i> strain VG <i>ribC</i> gene, partial cds
52	KR092386	0.0	100	<i>B. clarridgeiae</i> strain VG <i>ribC</i> gene, partial cds
57	CP020742	0.0	99.62	<i>B. henselae</i> strain Houston-I chromosome, complete genome
62	AY953284	0.0	99.81	<i>B. henselae</i> riboflavin synthase (<i>ribC</i>) gene, partial sequence
79	KR092386	0.0	100	<i>B. clarridgeiae</i> strain VG <i>ribC</i> gene, partial cds
80	KR092386	0.0	100	<i>B. clarridgeiae</i> strain VG <i>ribC</i> gene, partial cds
86	AY953284	0.0	99.80	<i>B. henselae</i> riboflavin synthase (<i>ribC</i>) gene, partial sequence
90	HM588661	0.0	100	<i>B. henselae</i> strain BART05 <i>ribC</i> gene, partial cds
95	CP020742	0.0	99.59	<i>B. henselae</i> strain Houston-I chromosome, complete genome
97	KR092386	0.0	99.81	<i>B. clarridgeiae</i> strain VG <i>ribC</i> gene, partial cds

3. Results

Table 8 (continued).

101	CP020742	0.0	99.80	<i>B. henselae</i> strain Houston-I chromosome, complete genome
107	AY953284	0.0	99.80	<i>B. henselae</i> riboflavin synthase (<i>ribC</i>) gene, partial sequence
108	KR092386	0.0	99.81	<i>B. clarridgeiae</i> strain VG <i>ribC</i> gene, partial cds
	AY953284	0.0	99.62	<i>B. henselae</i> riboflavin synthase (<i>ribC</i>) gene, partial sequence
119	CP020742	0.0	100	<i>B. henselae</i> strain Houston-I chromosome, complete genome
118	KR092386	0.0	100	<i>B. clarridgeiae</i> strain VG <i>ribC</i> gene, partial cds

3.2.4.3. Molecular cloning for detection of coinfection

The sequence obtained from sample number 108 presented overlapping nucleotides for both *gltA* and *ribC* genes and, because of this, was not possible to analyze with BLAST, as referred above; however, a coinfection with two or more species of *Bartonella* was suspected. In order to determine the possible coinfection, a molecular cloning was performed for both *gltA* and *ribC* amplicons for sample 108. The *gltA* amplified fragments from the positive colonies were almost indistinguishable after electrophoresis and even though they were sequenced. The result was undetermined. On the contrary, *ribC* colonies amplified DNA fragments that were positive (**Figure 8**).

3. Results

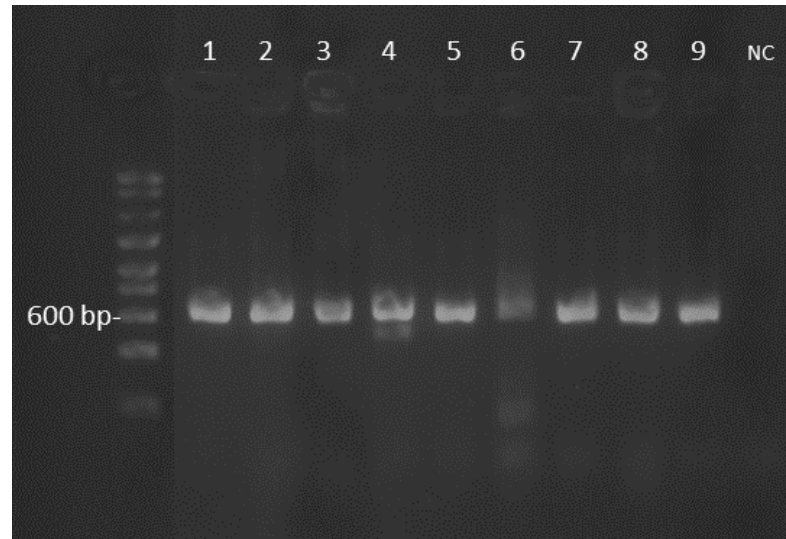


Figure 8. Electrophoresis in 1.5% agarose gel stained with 0.03 μL / mL of Green Safe Premium (Nzytech) showing the amplicons for *ribC* (585 bp) clone colonies from sample 108. (MW: molecular size marker; Numbers 1 to 9 correspond to the colonies; NC: negative control).

DNA extracted from the colonies numbered as 1, 2, 5, 7 and 9 were sequenced and compared using BLAST. The analysis confirmed that sample 108 presented a coinfection of both *B. henselae* and *B. clarridgeiae* (more details about BLAST analysis for sample 108 are listed on **Table 8**; 3.2.4.2).

3.2.5. Sequence analysis

3.2.5.1. Sequence analysis *gltA*

For the dataset of *B. henselae gltA* (13 sequences), analyzed using the DNA Sequence Polymorphism software (DNAsp), the number of sites found was 717, nucleotide diversity (Pi) was 0.000, haplotype diversity (Hd) of 0.000 and only one haplotype was identified under the GeneBank accession number MN564828.

Furthermore, for the dataset of *B. clarridgeiae gltA* (11 sequences), the number of sites reported by the DNAsp software was 743, nucleotide diversity (Pi) was of 0.000, haplotype diversity also of 0.000 and only one haplotype was identified under the GeneBank accession number MN564829.

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3.2.5.2. Sequence analysis *ribC*

Similarly, for the dataset of *B. henselae ribC* (14 sequences), analyzed using the DNA Sequence Polymorphism software, the number of sites found was 530. Sequences presented a nucleotide diversity (Pi) of 0.00081 and haplotype diversity (Hd) of 0.2747. Three haplotypes were identified: haplotype 1 (Hap1, GeneBank accession number MN564824), corresponding to 12 samples (2, 16, 27, 29, 37, 57, 62, 86, 90, 107, 108, and 119); haplotype 2 (Hap2, GeneBank accession number MN564825), corresponding to sample 95, and haplotype 3 (Hap3, GeneBank accession number MN564826), corresponding to sample 101.

Regarding the *B. clarridgeiae* group (11 sequences), the number of sites found and analyzed by the DNAsp software were 521. The nucleotide diversity (Pi) reported was 0.00035, and the haplotype diversity 0.182. Two haplotypes were identified: Haplotype 1 (Hap1, GeneBank accession number MN564827), corresponding to 10 samples (19, 22, 28, 33, 46, 52, 79, 80, 97, and 118), and Haplotype 2, corresponding to sample 108 (Hap2, GeneBank accession number MN632451).

3.2.6. Phylogenetic Analysis

Both Maximum Likelihood (ML) and Neighbor Joining (NJ) phylogenetic trees for *gltA* were alike, being the most notorious difference the position of the *B. clarridgeiae* clade, located closer to the outgroup (*Brucella abortus*) when constructed by ML method. The unique haplotype for *B. henselae* (MN564828) was grouped on the same cluster with other *B. henselae* from different countries and reservoirs, including one isolated from cats, in Brazil (MH019304.1), and cat fleas, in Austria (MF374384.1), supported by a bootstrap value of 99% for ML and 100% for NJ. In addition, the unique haplotype for *B. clarridgeiae* (MN564829) was grouped on a cluster together with other *B. clarridgeiae* strains, including two isolations from cats in Brazil (MH019302.1) and Thailand (KX001761.1) and one from cat fleas from Chile (KY913636.1), supported by a 100% bootstrap value for both ML and NJ methods. (**Figures 9 and 10**).

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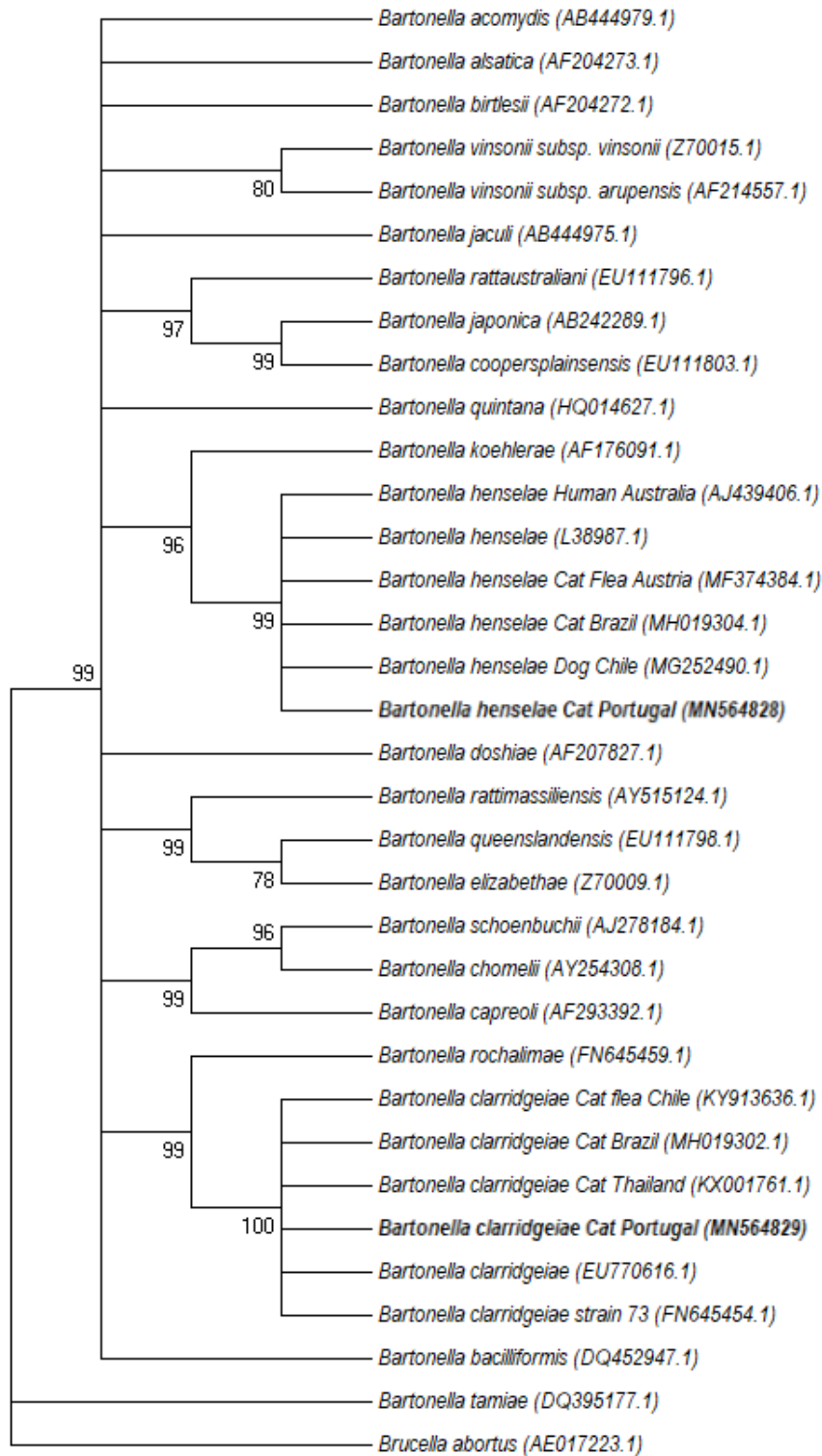


Figure 9. Phylogenetic analysis of *gltA* sequences (296 bp after alignment) based on the Maximum Likelihood method and model Kimura-2-Parameters. Numbers are corresponding to the support values for a bootstrap with 1000 repetitions, and only bootstraps > 70% are presented. *Brucella abortus* was used as outgroup.

3. Results

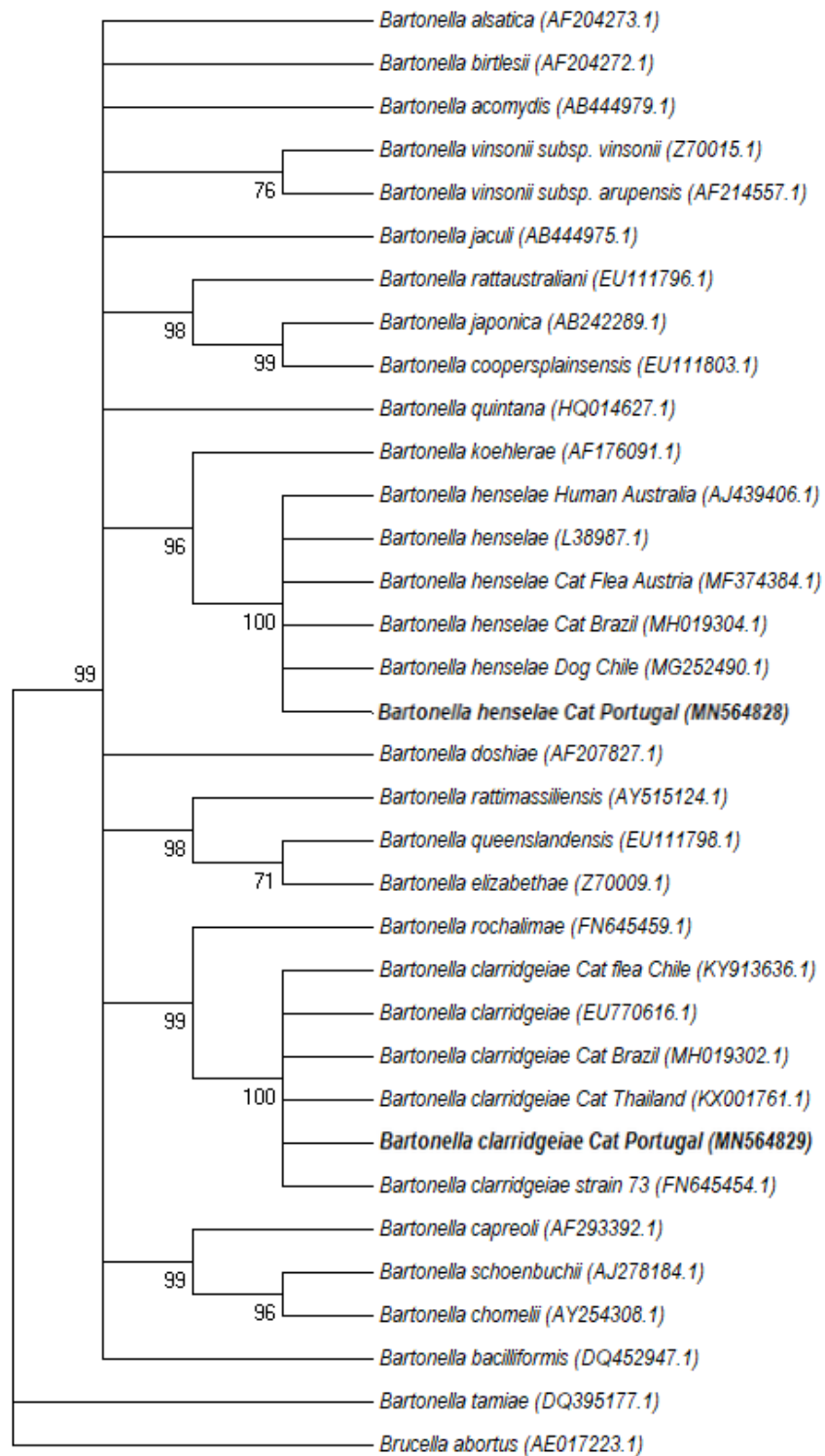


Figure 10. Phylogenetic analysis of *gltA* sequences (296 bp after alignment) based on the Neighbor Joining method and model Kimura-2-Parameters. Numbers are corresponding to the support values for a bootstrap with 1000 repetitions, and only bootstraps > 70% are presented. *Brucella abortus* was used as outgroup.

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Phylogenetic analysis for gene *ribC* showed a similar distribution for both Maximum Likelihood (ML) and Neighbor Joining (NJ) Method. All tree haplotypes for *B. henselae* (MN564824, MN564825 and MN564825) was grouped on the same clade together with sequences corresponding to other *B. henselae* isolated from cats in Brazil (HM588661) and fleas in the USA (AY953284); in addition, the referred cluster had a 100% bootstrap support for the branch, for both ML and NJ methods. Furthermore, the two haplotypes for *B. clarridgeiae* (MN564827 and MN632451) formed a cluster supported for 100% bootstrap, together with *B. clarridgeiae* sequences isolated from cats from Brazil (KR092386), Japan (AB292604) and China (EU571943). Both clusters are similar for both ML and NJ methods. (**Figure 11** and **12**).

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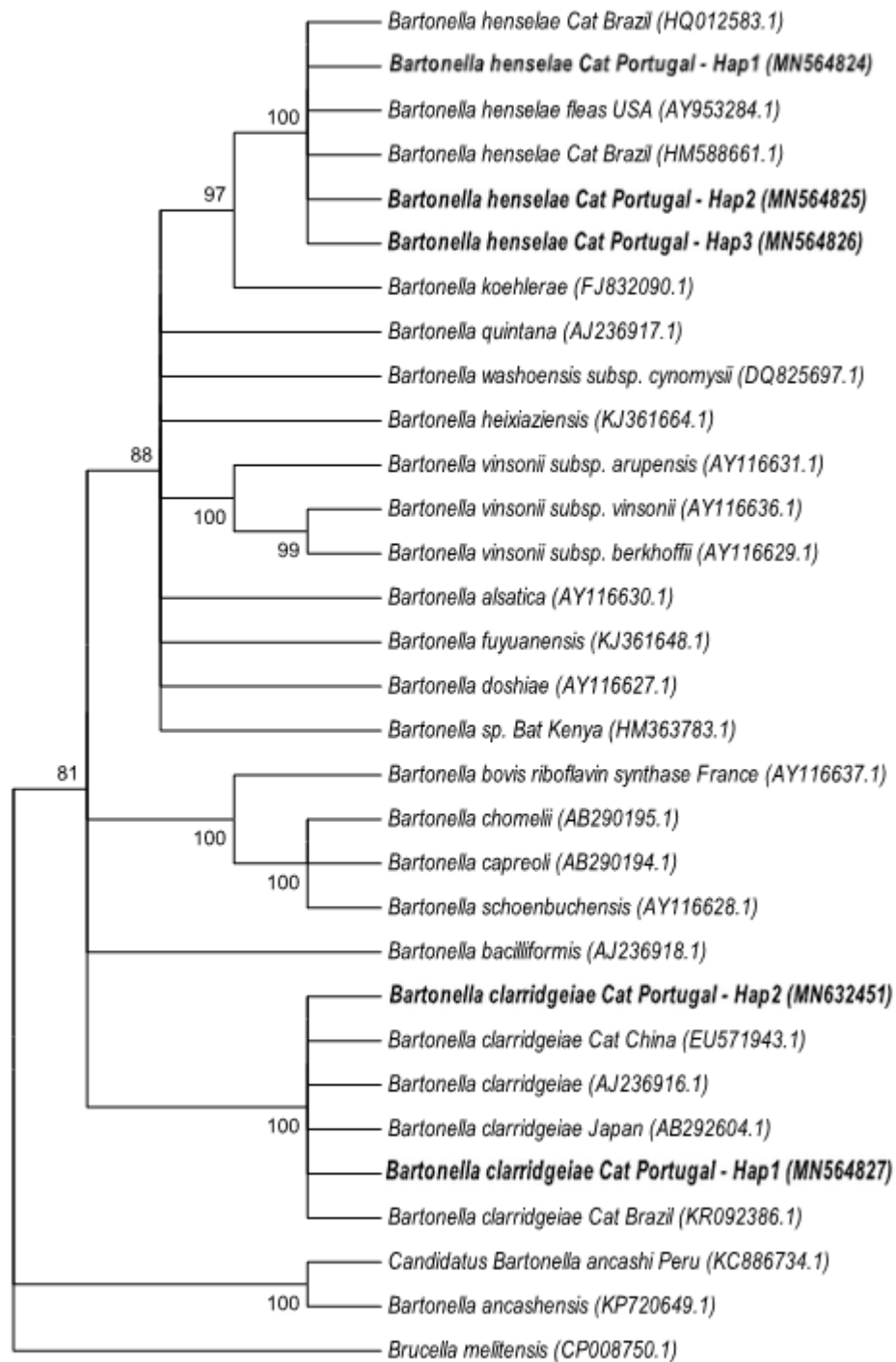


Figure 11. Phylogenetic analysis of *ribC* sequences (455 bp after alignment) based on the Maximum Likelihood method and model Kimura-2-Parameters. Numbers are corresponding to the support values for a bootstrap with 1000 repetitions, and only bootstraps > 70% are presented. *Brucella abortus* was used as outgroup.

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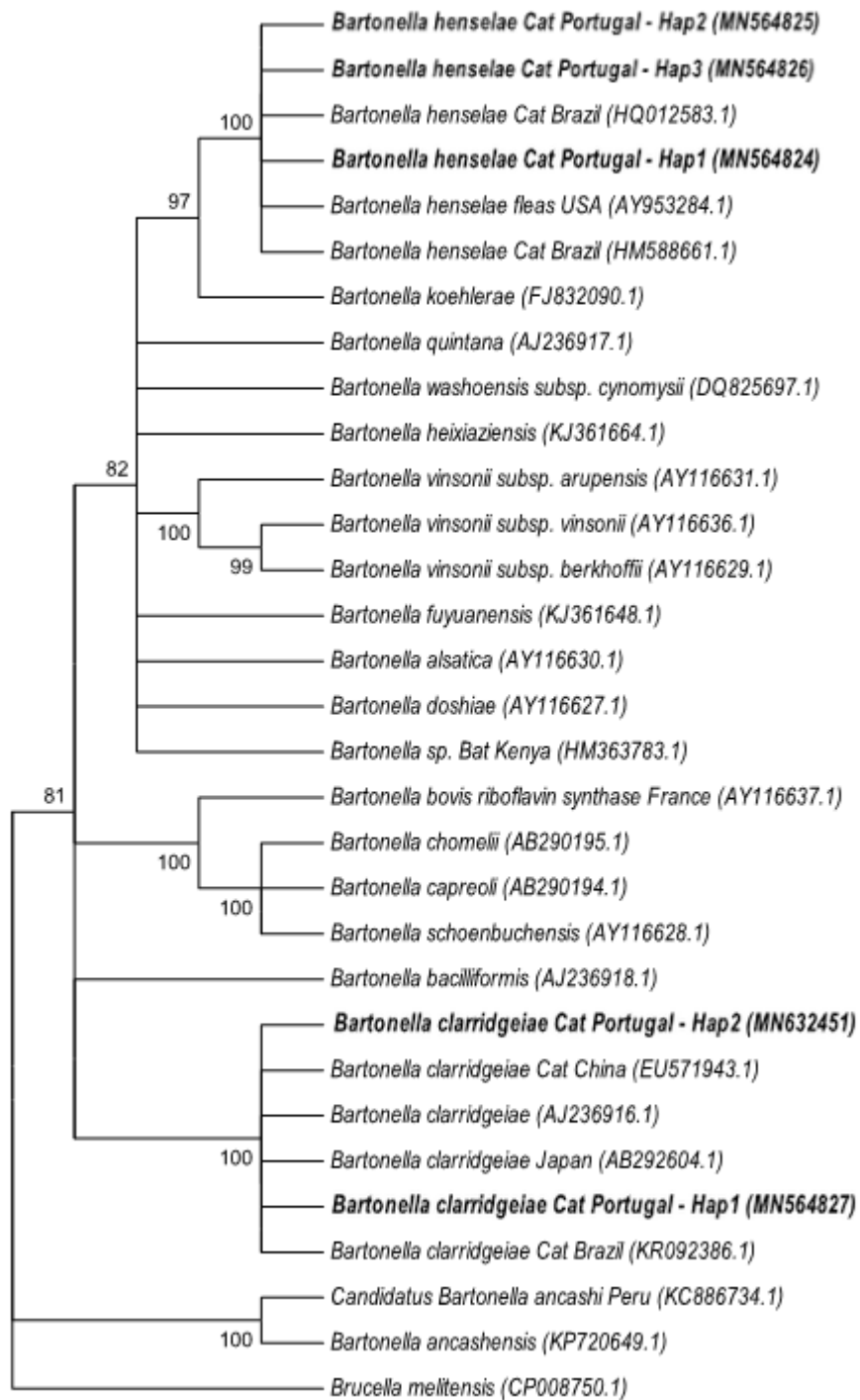


Figure 12. Phylogenetic analysis of *ribC* sequences (455 bp after alignment) based on the Neighbor Joining method and model Kimura-2-Parameters. Numbers are corresponding to the support values for a bootstrap with 100 repetitions, and only bootstraps > 70% are presented. *Brucella abortus* was used as outgroup.

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3.3. Dogs

3.3.1 Sample collection and DNA extraction

From a total of 25 samples coming from 15 different geographical points in Lisbon, distributed in 12 Lisbon locals, 11 (47.82%) corresponded to adult dogs and 12 (52.17%) to juveniles. Furthermore, 10 (43.47%) were female and 15 (56.52%) were male. Only four (17.39%) were flea infested. Two dogs did not have any records (corresponding to samples 22 and 23) (**Annex 4**).

DNA extracted from all the 25 samples were quantified by Nanodrop spectrophotometer, presenting concentrations ranged from 4.4 ng/ μ L to 88.6 ng/ μ L.

3.3.2. DNA integrity confirmation

DNA extracted from the samples were tested for *GAPDH* (mammalian endogenous gene) in order to confirm the DNA integrity. **Figure 13** shows the first group of samples tested, all positive by a cPCR targeting *GAPDH* gene. The same results were obtained for the remaining samples.

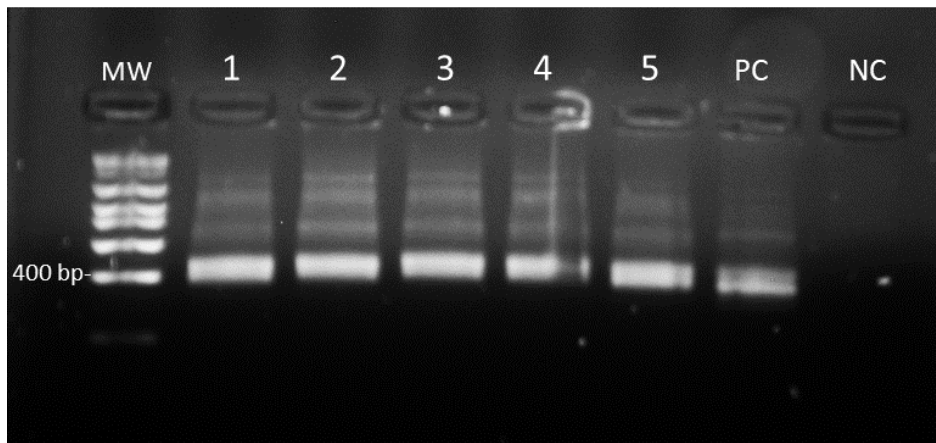


Figure 13. Electrophoresis in 1.5% agarose gel stained with 0.03 μ L / mL of Green Safe Premium (Nzytech) showing the dog DNA amplicons for a fragment of 437 bp of the *GAPDH* gene. (MW: molecular weight size marker; wells 1-5 corresponding to samples 1-5 respectively; NC: negative control).

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3.3.3. Molecular detection for *Bartonella* sp.

All dog samples were negative for the qPCR targeting *nuoG* gene. The mean efficiency (E) of the qPCR reactions was 95.25% (90.1% to 100.4%), and the slope ranged from -3.583 ($R^2= 0.999$) to -3.313 ($R^2=0.994$). Therefore, a molecular characterization was not performed.

3.4. Humans

3.4.1 Sample collection and DNA extraction

During this study, 30 human blood samples were collected, DNA extracted and subsequently, quantified using a Nanodrop spectrophotometer. DNA concentrations ranged from 10.3 ng/ μ L to 197.1 ng/ μ L.

3.4.2. DNA integrity confirmation

The obtained thirty samples were tested for *GAPDH* gene in order confirm the integrity of the extracted DNA, as done for the other samples. **Figure 14** shows amplicons for the first group of five samples. The same results were obtained for the remaining samples.

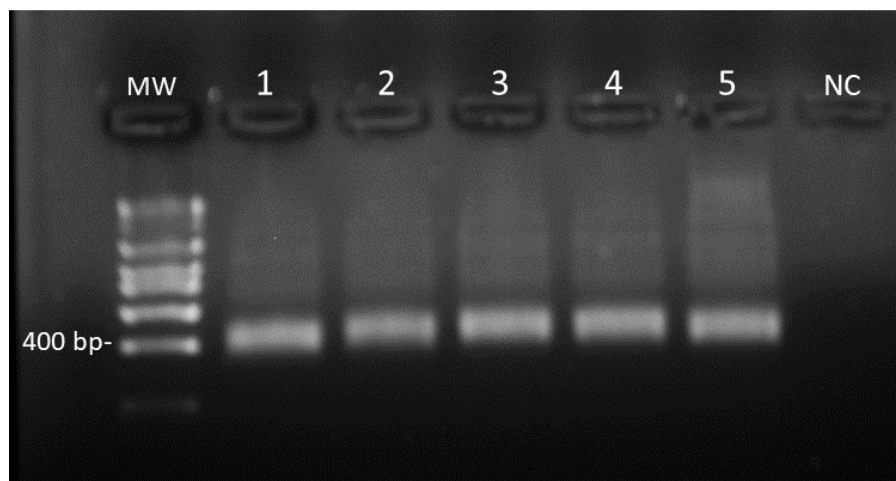


Figure 14. Electrophoresis in 1.5% agarose gel stained with 0.03 μ L / mL of Green Safe Premiun (Nzytech) showing human DNA amplicons for a fragment of 437 bp of the

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GAPDH gene. (MW: molecular weight size marker; wells 1-5 correspond to samples 1 to 5, respectively; NC: negative control).

3.4.3. Molecular detection for *Bartonella* sp.

All 30 human samples were negative for the qPCR targeting *nuoG* gene. The mean efficiency (E) of the qPCR reactions was 106.3% (100.4% to 112.2%), and the slope ranged from -3.313 ($R^2= 0.994$) to -3.061 ($R^2=0.363$). Therefore, a molecular characterization was not performed.

3.5. Cultures

3.5.1. Liquid cultures DNA extraction and DNA integrity

After 10 days of incubation in BAPGM liquid medium, DNA extraction of 55 cultures (25 corresponding to dog samples and 30 to human samples) were performed and DNA screened with a cPCR for *GAPDH* in order to confirm DNA integrity, as previously described. From the 25 samples coming from dog blood cultures, only 15 (corresponding to dog specimens number 1, 5, 6, 8, 9, 10, 11, 12, 14, 18, 19, 20, 21, 23 and 24) were positive for *GAPDH*. In contrast, all DNA samples from cultured human blood were positive for *GAPDH* (**Figure 15**).

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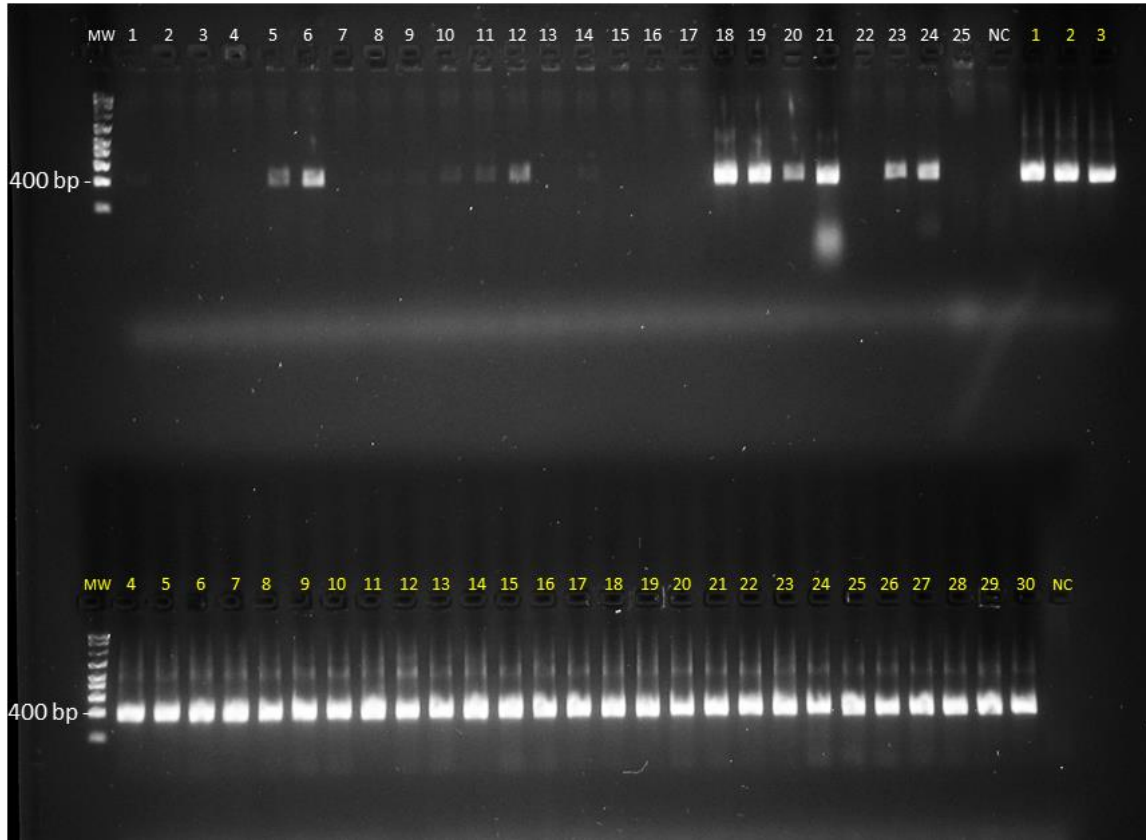


Figure 15. Electrophoresis in 1.5% agarose gel stained with 0.03 μL / mL of Green Safe Premium (Nzytech) showing the amplicons for a fragment of 437 bp of the *GAPDH* gene. (MW: molecular weight size marker; wells 1 - 25 in white color: dog DNA culture samples 1 to 25 respectively; wells 1 - 30 in yellow color: human DNA culture samples 1 to 30 respectively; NC white/yellow: negative control)

3.5.2. Solid culture follow-up

For each dog and human sample, positive for the *GAPDH* cPCR, two cultures in solid medium (a direct one from blood and a subculture of the BAPGM liquid culture) were performed, giving a total of 110 plates. From these, only in 11 was possible to observe colony growing (**Table 9**) and only 2 (corresponding to dog specimen number 2 and 21) showed colonies with morphological or behavioral characteristics typical from *Bartonella* sp. (**Figure 15**).

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Table 9. Cultures dates of appearance and their morphological characteristics.

Culture	First date of Incubation	Date in which colony emerged	Days for colony to turn visible	Morphology
2D	25/06/19	10/07/19	15	Red color
18D	19/07/19	23/07/19	4	Yellow, medium circles
21D	19/07/19	21/07/19	2	Variable
21Dsub	29/07/19	05/08/19	8	Small, circular
22D	19/07/19	23/07/19	4	Yellow, medium circles
22Dsub	29/07/19	28/08/19	30	White big circle
6H	08/08/19	16/08/19	8	Branches, fungus like
17H	08/08/19	12/08/19	4	Big white circles
24H	18/08/19	28/08/19	10	Big white spot
27H	18/08/19	28/08/19	10	Yellow, medium circles
29Hsub	27/08/19	28/08/19	1	Yellow, medium circles

Suffix D: dog blood cultures, suffix H: human blood cultures; suffix sub: subculture)

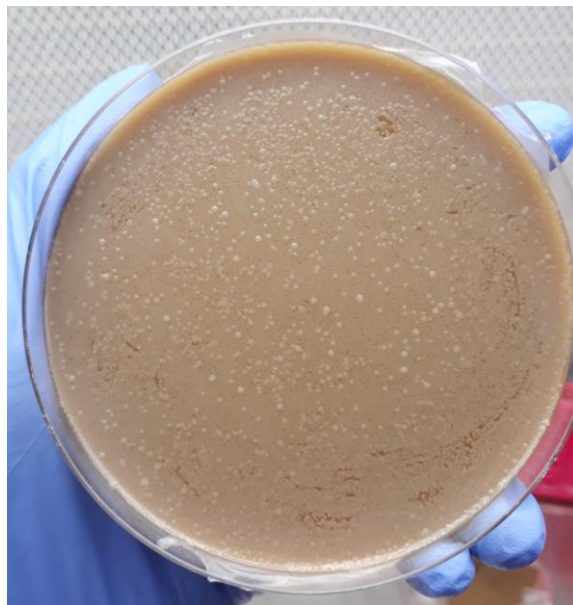


Figure 16. Subculture 21 from dog blood. Conformed by small circular colonies, typical for *Bartonella* sp.

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3.5.3. DNA integrity confirmation

Culture 2D and 21Dsub were selected because of their behavioural and morphological characteristics to be screen for *Bartonella* sp. DNA. After DNA extraction, a cPCR for gene *ribC* was carried out in order to confirm integrity of bacterial DNA (Figure 16). Only DNA sample 2D was positive for *ribC*.

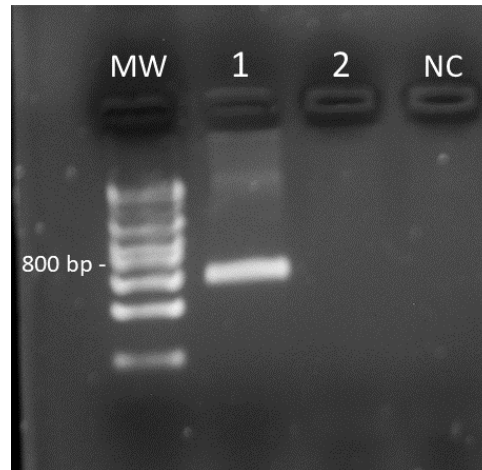


Figure 17. Electrophoresis in 1.5% agarose gel stained with 0.03 μL / mL of Green Safe Premiun (Nzytech) showing the amplicons for *ribC* gene (585 bp) for DNA from cultured dog blood samples. MW: molecular weight size marker; well 1: 2D; well 2: 21Dsub; NC: negative control).

3.5.4. Molecular detection for *Bartonella* sp.

All 45 DNA samples from liquid cultures (15 from dog blood and 30 from human blood, referred in 3.5.1.) and DNA from solid culture 2D (from dog specimen number 2) were negative for the qPCR targeting *nuoG* gene. The mean efficiency (E) of the qPCR reactions was 95.85% (92.3% to 99.4%), and the slope ranged from -3.483 ($R^2= 0.998$) to -3.215 ($R^2=0.999$). Thus, molecular characterization was not performed.

In addition, a purified amplicon for *ribC* gene of the 2D sample was sequenced. The BLAST analysis showed a 97.7% of identity with *Staphylococcus epidermidis* strain HD33 (GeneBank accession number CP040864), confirming that the amplified product

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for *ribC* was not from *Bartonella* sp. DNA. The sequenced sample was considered, therefore, a false-positive.

4. Discussion and conclusions

4. Discussion and conclusions

Portugal, as a Mediterranean country, possess favorable temperatures and humidity levels for flea and tick infestations which may facilitate *Bartonella* spp. infections in domestic animals or even people, however, only few studies have been performed on the topic (Álvarez-Fernández et al., 2018). As a consequence, the present study tried to assess the prevalence of *Bartonella* sp. in its mainly hosts and reservoirs: cats, dogs and humans, and a potential vector: ticks.

In regards to cats, the present study reports 20.32% of positive samples for *Bartonella* sp. (detected by a qPCR targeting *nuoG*), 13 characterized as *Bartonella henselae*, 11 as *Bartonella clarridgeiae* and one that presented a co-infection of both species (by two cPCR targeting *gltA* and *ribC*). The percentage of positive samples was higher than the study performed in southern Portugal by Maia et al. in 2014, where only 2.9% of cats were positive for *Bartonella* sp. using a cPCR targeting a fragment of *ITS* gene. On the other hand, lower than the one performed in Évora in 2009 by Alves et al., which reported a higher prevalence for *Bartonella henselae* in cats (67.7% by cPCR targeting a fragment of *gltA* gene). The different prevalence levels reported by these two past studies and the current one may be discussed considering many factors. Firstly, half of the animals surveyed by Maia et al. (2014) were domestic cats living indoors and not stray cats, like in the present study; this could explain the difference on the prevalence levels found (2.9% vs. 20.32%) due to a higher probability of flea infestation on stray cats than in domestic ones. Secondly, Alves et al. (2009) investigation was carried out on cats from Évora, a city with an average summer temperature of around 30 °C and also one of the cities of Portugal that has increased the average temperature of the country for the last two decades with 0.8 °C, with values above the city of Lisbon (Costa, 2019). In addition, Évora is prone to severe heat extremes with an all-time record of 46 °C (Climate normals 1981-2010 – Évora, 2015) and this hot-summer Mediterranean climate is favorable for vector infestation in cats, which make them prone to *Bartonella* sp. infections. Finally, the molecular methods employed by all three studies are different, being the prior ones cPCR targeting *ITS* (Maia et al., 2014) and *gltA* (Alves et al., 2009) and the present investigation used a qPCR targeting *nuoG* followed by two cPCR for *gltA* and *ribC* gene for *Bartonella* spp. characterization. The present study was the first in the region to use this method to survey and quantify *Bartonella* sp. DNA. Nevertheless, the species of the genus *Bartonella* detected by all three studies were the same two: *B. henselae* and *B.*

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clarridgeiae, so it is possible to suggest that these two *Bartonella* species are the most abundant in cats from Portugal. Furthermore, the high levels of prevalence is quite worrisome for the region, because of a reported increase in the number of pet cats within the country, that went for an average of 100 thousands for 2010 to 1500 thousands reported for 2018 (Sabanoglu, 2019), which represents a higher risk for *Bartonella* sp. infection on cat owners from Portugal.

In terms of the phylogenetic findings, all sequences analyzed for both *B. henselae* and *B. clarridgeiae* were intimately close to other isolates from other parts of the world and even some were isolated not from cats, but from other mammals and vectors. As an example, for *gltA* sequences, *B. henselae* genotypes were close related to other *B. henselae* isolated from cats in Brazil (Silva et al., 2019), dogs in Chile (Muller et al., 2018), cat fleas in Austria (Duscher et al., 2018) and humans in Australia (Dillon et al., 2002). This proximity also took place for *B. clarridgeiae* and other isolations of *B. clarridgeiae* from cats in Brazil (Silva et al., 2019), Thailand (Srisanyong et al., 2016) and cat fleas from Chile (Muller et al., 2017). In addition, a similar phenomenon occurred for *ribC* sequences. It could be suggested that there are strains of *B. henselae* and *B. clarridgeiae* that are being shared by animals from different locations, possibly due to human and animal migration.

Despite the high prevalence detected for *Bartonella* sp. in cats from Lisbon, the results for dogs were all negative. A comparison with other studies is difficult to make, because most studies have relied on serology and have not used molecular methods (Chomel et al., 2006). The few that have used them relied on cPCR rather than a qPCR like in the current assay, being the closest possible comparison a study performed in Spain by Roura et al. in 2018 that uses a qPCR targeting *ITS*, however, the sample of dogs selected presented a prior diagnosis of blood culture-negative endocarditis based on consistent clinical signs, contrary to the dogs sampled from the present study, that were healthy or asymptomatic. Most of the time, dogs are only an incidental host for *Bartonella* spp. and not a main reservoir (Álvarez-Fernández et al., 2018), thus, a low level of bacteremia in healthy dogs could be below the sensitivity limits of the qPCR for *nuoG* for *Bartonella* sp. detection in dog blood. This could be the reason of why the dogs were negative for the assay. For that reason, the importance of performing blood cultures for

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Bartonella sp. molecular detection have already been stressed by other studies for dogs and humans (Roura et al., 2018 and Lantos et al., 2014); however, cultured samples were also negative for the present investigation. Nevertheless, *B. henselae* and *B. clarridgeiae*, *Bartonella* species that have been identified in cats during the present study, have also been reported in domestic dogs (Mosbacher et al., 2011 and Boulouis et al., 2005, respectively). Consequently, it is necessary to perform more studies, having into account the selection of a more diverse group of dogs, in terms of their medical condition, in order to have better insights about the *Bartonella* sp. prevalence in dogs from the region.

In Europe, *Bartonella* sp. infections affecting humans are starting to become more common and the bacteria is considered an emerging pathogen in the continent (Vorou et al., 2007). Prevalence rates have a trend to be higher in immune-compromised people (Maurin et al., 1996 and Koehler et al., 2003) and some species that cause endocarditis are associated with other specific risk factors, depending on the *Bartonella* spp. that causes the disease. For example, *B. quintana* infection is associated with homelessness, alcoholism, and body louse infestation, while *B. henselae* infection is associated with cat contact and cat flea exposure (Fournier et al., 2001). Moreover, veterinary personnel would be included in this last group of risk due to their constant contact with potential *Bartonella* sp. infected animals and surveys to screen *Bartonella* sp. on this risk group have already been performed both in Europe and out of Europe: Lantos et al. (2014) detected *Bartonella* sp. DNA in 32 out of the 114 veterinary subjects (28%) by a cPCR for *ITS* gene, in the same fashion; Oteo et al. (2017) detected the bacteria in Spain on 27% of the sampled individuals. It is important to point on the fact that both works carried out the *Bartonella* sp. screening on cultured blood, however, during the present study no samples from any humans, both for the assays carried out directly on extracted DNA from blood and after blood culture, were positive for *Bartonella* sp. Despite the 113 cases reported to date for infections with *B. quintana*, *B. henselae*, *B. elizabethae*, and *B. vinsonii* subsp. *berkhoffii*, associated with infective endocarditis in humans in Europe (Zeaiter et al., 2003), not many cases have been reported for Portugal. However, some recent cases have been reported in previously healthy people, the first one involving an adolescent with right pre-auricular adenopathy that reported contact with a small cat; *B. henselae* was detected by serology (Dias et al., 2012). The other case involved a previously healthy 29-year-old man with lymphadenopathy in the axillary, *Bartonella*

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spp. being detected in his blood and cutaneous lesions biopsy (Costa et al., 2011). There are also some reports involving immune-compromised people, such as the case of an HIV patient with bacillary angiomatosis in which *Bartonella quintana* infection that was detected by cPCR (Santos et al., 2000), the case of a 73 year-old woman with chronic heart condition that presented endocarditis caused by a *Bartonella* sp. detected by serology (Cordero et al., 2008) and a 44-year-old woman, who was a kitten owner, presenting a papule in a finger of the right hand and regional lymphadenopathies, whose diagnosis was performed after detection of *Bartonella* sp. DNA by serology and a PCR on lymph node biopsy (Murinello et al., 2010). Having into account the most recent cases, during the present study we selected 30 individuals that work in close contact with cats during a big part of their daily routines that accepted to participate in the study. Blood samples that were tested directly (after DNA extraction) and after culture in liquid and solid medium were negative for all 30 individuals, as in contrast with the *Bartonella* sp. DNA found in cats in Lisbon (20%). It is possible that the bacteremia levels (if there was any) were lower than the minimum concentration required for the *nuoG* qPCR to be able to detect the bacteria, taking into account that all individuals were asymptomatic. However, even after blood culture was performed for each sample, *Bartonella* sp. DNA was not detected, which would suggest that all sampled humans were free from *Bartonella* spp. infection. Finally, it is important to mention that this is the first study carried out in Portugal to screen *Bartonella* sp. in asymptomatic humans with a qPCR targeting *nuoG* gene. Nevertheless, more studies need to be carried out, involving other molecular methods and groups of individuals known to have been in contact with an infected cat and that are also immune-compromised, such as patients already diagnosed with HIV, autoimmune diseases, cancer, or pregnant women.

In regards of the main tick species in Europe (and that were also identified during the present study), there has been 8 previous reports of *Bartonella* sp. in *Ixodes ricinus* by cPCR methods targeting *groEL*, *pap31*, *ftsZ* (Sanogo et al., 2003), *gltA* (Podsiadly et al., 2007), *16S rRNA* (Schouls et al., 1999) and *ITS* (Bogumila et al., 2005). In contrast, there are only two reports for *Bartonella* sp. detection in *Rhipicephalus sanguineus* that were carried out targeting *ribC* (Wikswow et al., 2007), *ITS* and *nuoG* (Billeter et al., 2008) genes. In the present study, however, *Bartonella* sp. was not detected for any of the 268 collected ticks (114 *Ixodes ricinus*, 18 *Ixodes* sp. and 136 *Rhipicephalus sanguineus* sensu

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lato), despite having a large number of specimens, whose distribution englobed eight districts of Portugal territory. *Bartonella* sp. has been previously detected on ticks by a molecular method targeting *nuoG* (Billeter et al., 2008), although it is important to mention that detection on that study was performed by using a cPCR based method and not a qPCR, like in the current investigation. It is possible however, that *Bartonella* sp. DNA may have not been present on the sample of ticks studied, which would suggest that ticks are not a potential *Bartonella* sp. vector on the region. Nevertheless, more surveys with a bigger sample and maybe multiple detection methods should be carried out in the future to address this possibility.

4.1. General conclusions

About 20% of cats sampled in Lisbon were positive for at least one *Bartonella* spp. In addition, both *B. henselae* and *B. clarridgeiae* were identified in similar percentages (52% and 48% respectively). It is hoped that these results could bring attention to *Bartonella* sp. as an emerging pathogen in cats from Lisbon, before the bacteria represents a bigger issue for public health in Portugal, taking into account the increasing number of domestic and stray cats in the region (Sabanoglu, 2019).

Despite *Bartonella* spp. were not detected in ticks, dogs and humans during the present study, more studies need to address on this topic, putting emphasis in *Bartonella* spp. reservoirs and vectors to have a better understanding of the bacteria and the general prevalence levels within the population in order to prevent or stop further transmission.

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6. Annexes

6. Annexes

Annex 1. Committee on the Ethics of Animal Experiments from the Institute of Hygiene and Tropical Medicine (CEIHMT) approval of this study under the permit 01.19.



Conselho de Ética
Instituto de Higiene e Medicina Tropical
Universidade Nova de Lisboa

Parecer 01.19

Título do Estudo:

Deteção molecular de Bartonella spp. em humanos, animais domésticos e ectoparasitas amostrados em Lisboa, Portugal

Investigador principal:

Ana Domingos

Outro(s) Investigador(es) / Orientadores:

Sandra Antunes, Gustavo Seron Sanches, Joana Ferrolho, Estefania Torrejon Navarro, Lenira Santos

Entidade promotora:

Instituto de Higiene e Medicina Tropical

Entidade onde se efetua o estudo:

Instituto de Higiene e Medicina Tropical

Objetivos/Resumo do estudo:

Este estudo tem como finalidade identificar as espécies de Bartonella circulantes em animais domésticos (cães e gatos), ectoparasitas (carrapatos e pulgas) e em seres humanos, afim de contribuir para o melhor entendimento do ciclo da doença, bem como para facilitar e contribuir com diagnóstico no caso de infeções. O objetivo geral consiste na deteção molecular de Bartonella spp. em seres humanos, animais domésticos (cães e gatos) e em ectoparasitas (pulgas e carrapatos) amostrados em Lisboa, Portugal em 2019. São objectivos específicos, 1) Identificar molecularmente as espécies de Bartonella encontradas; 2) caracterizar filogeneticamente as espécies encontradas; 3) Calcular a frequência de cada espécie em cada grupo em estudo.

6. Annexes

Conflito de Interesses:

Não existe conflito de interesses.

Consentimento informado, informação para o participante e confidencialidade:

O consentimento informado está de acordo com as normas de elaboração destes documentos.

Conclusão:

- Parecer Favorável
- Parecer Provisório
- Parecer Não Favorável

Documentos analisados:

- Protocolo datado de 19/12/2018 ou versão
- Questionário do estudo
- Formulário de informação do estudo e consentimento informado
- Guião de entrevista do estudo
- CV dos investigadores externos ao IHMT
- Outras autorizações relevantes

Lisboa, 21/01/2019


Professora Doutora Filomena Pereira
Presidente do Conselho de Ética

6. Annexes

Annex 2. Informed consent for volunteered participation as blood donors for the present study

CONSENTIMENTO INFORMADO

Título do Estudo: Detecção molecular de *Bartonella* spp. em humanos, animais domésticos e ectoparasitas amostrados em Lisboa, Portugal

Promotor: Instituto de Higiene e Medicina Tropical / Universidade Nova de Lisboa – Unidade de Ensino e Investigação (UEI) de Parasitologia Médica

Investigadores: Estefania Torrejón Navarro (Mestranda), Ana Domingos (PhD) e Gustavo Seron Sanches (PhD)

Contatos e-mail investigadores: adomingos@ihmt.unl.pt, gustavo.seron@ihmt.unl.pt

Contacto: Rua da Junqueira, nº.100, 1349-008 Lisboa

Telefone: 21 365 26 61

Fax: 21 363 21 05

INTRODUÇÃO

As bartonelas são um grupo de bactérias que podem causar diversas enfermidades, dentre as quais, algumas podem acometer humanos, como é o caso da doença da arranhadura do gato. O gato doméstico, pode se infetar por meio da mordedura por pulgas e consequentemente transmitir o patógeno a humanos por meio da mordida ou da arranhadura. Embora menos frequente, as carraças poderiam transmitir a bactéria aos gatos e também diretamente aos humanos. Tanto o homem como os animais domésticos podem conviver de forma assintomática com estas bactérias, entretanto, em alguns casos pode haver a manifestação de forma mais severa da doença, que pode ser potencialmente fatal, se não tratada. Além disso, o diagnóstico da doença por vezes torna-se complicado, onde cada caso pode apresentar uma série de sintomas atípicos e que podem se confundir com outras doenças, o que justifica a importância deste tipo de estudo.

Apresenta-se em seguida toda a informação sobre o estudo, para poder decidir se quer ou não participar. Esta é uma decisão pessoal. Se tiver alguma questão ao ler este documento, pode colocá-la a quem lho forneceu, e contactar qualquer um dos investigadores.

A) OBJECTIVO DO ESTUDO

Esta doença pode ser confundida com outras doenças, devido à semelhança das manifestações clínicas, ou mesmo à ausência de sintomas. Os estudos publicados sobre esta doença em Portugal são ainda poucos. Pretendemos saber se as pessoas possuem esta bactéria, mesmo que não tenham sintomas.

B) PROCEDIMENTO DO ESTUDO

Se aceitar fazer parte deste estudo terá apenas que preencher o termo que segue. Para saber se já foi infetado(a) vamos precisar de colher uma pequena parte de sangue (1mL). Mais tarde a amostra será encaminhada para o Laboratório de Biologia Molecular da UEI de Parasitologia Médica do Instituto de Higiene e Medicina Tropical (IHMT), onde será feita a análise. Se der a sua autorização, será contactado se o resultado for positivo, para que possa conversar com o seu médico assistente e decidir se deverá fazer algum tratamento.

C) PARTICIPAÇÃO VOLUNTÁRIA E LIBERDADE PARA DESISTIR

É totalmente sua a decisão de participar ou não. Se não quiser participar não haverá prejuízo para si.

D) RISCOS E DESCONFORTOS

Para participar neste estudo, o risco e desconforto que existe é mínimo, ligado apenas à colheita de 1mL de sangue.

E) BENEFÍCIOS

Para si haverá benefício caso seja detetada a presença de *Bartonella* em seu sangue. (esteja infetado) pois será avisado e poderá ser tratado pelo médico assistente.

Para a população, o benefício que há neste estudo é permitir que se conheça a distribuição da doença, em indivíduos assintomáticos residentes em Portugal e alertar as autoridades de saúde.

F) COMPENSAÇÃO

Não terá que pagar nada para fazer parte deste estudo, nem será pago para tal.

6. Annexes

G) CONFIDENCIALIDADE

As suas informações são confidenciais.

Os questionários vão ter um código e não o seu nome. Apenas o investigador terá acesso aos nomes correspondentes aos códigos, que serão guardados por ele. Toda a informação relativa às amostras e aos dados será guardada por um prazo máximo de 2 anos e após este período serão destruídos.

H) PUBLICAÇÃO DOS RESULTADOS

Os resultados deste estudo serão comunicados às autoridades de saúde, mostrados em reuniões científicas e publicados em revistas científicas, mas não terão dados confidenciais.

6. Annexes

I) DECLARAÇÃO DA PARTICIPANTE

Fui convidado a participar no estudo sobre Bartonelose, e sei que envolve a utilização de uma amostra de sangue obtida na colheita que realizo neste serviço. Fui informado de que os riscos são mínimos, e sei que os benefícios diretos só existem se estiver infetado. Foi-me dado o nome do investigador com quem posso contactar. Li toda a informação deste documento, ou foi-me lida. Tive oportunidade de fazer perguntas sobre o estudo. Autorizo voluntariamente a minha participação neste estudo, e sei que tenho liberdade para desistir a qualquer altura, sem prejuízo para os meus cuidados de saúde neste Instituto.

Nome do participante:

Contacto telefónico: _____

Código: _____

Data: __/__/____

Assinatura do participante (cruz se iletrado)

Testemunha (caso o participante seja iletrado):

6. Annexes

Annex 3. Breed, sex, age, local of origin, and presence of flea infestation concerning cats whose blood was donated by CAL.

Samples	Breed	Sex	Age	Local of origin	Flea infested
1	European Shorthair	Female	Adult	Campo de Ourique	WI
2	European Shorthair	Male	Adult	Marvila	No
3	European Shorthair	Female	Adult	Alcântara	No
4	European Shorthair	Male	Adult	Marvila	No
5	European Shorthair	Female	Adult	Belém	No
6	European Shorthair	Female	Adult	Estrela	No
7	European Shorthair	Male	Adult	Benfica	No
8	European Shorthair	Female	Adult	Misericórdia	No
9	European Shorthair	Female	Juvenile	São Domingos de Benfica	No
10	European Shorthair	Female	Adult	São Domingos de Benfica	No
11	European Shorthair	Female	Adult	São Domingos de Benfica	Yes
12	European Shorthair	Female	Adult	São Domingos de Benfica	No
13	European Shorthair	Female	Adult	São Domingos de Benfica	No
14	European Shorthair	Female	Adult	Misericórdia	No
15	European Shorthair	Female	Adult	São Domingos de Benfica	No
16	European Shorthair	Male	Adult	Estrela	No
17	European Shorthair	Female	Adult	Estrela	No
18	European Shorthair	Female	Adult	Alcântara	No
19	European Shorthair	Male	Adult	Alcântara	No
20	European Shorthair	Male	Adult	Campolide	No

6. Annexes

Annex 3 (continued).

21	European Shorthair	Female	Adult	Misericórdia	No
22	European Shorthair	Male	Adult	Misericórdia	No
23	European Shorthair	Female	Adult	São Domingos de Benfica	No
24	European Shorthair	Female	Adult	Campolide	No
25	European Shorthair	Female	Adult	São Domingos de Benfica	No
26	European Shorthair	Male	Adult	Arroios	Yes
27	European Shorthair	Female	Juvenile	Campolide	Yes
28	European Shorthair	Male	Juvenile	Penha Franca	No
29	European Shorthair	Female	Juvenile	Campolide	Yes
30	European Shorthair	Male	Adult	Ajuda	No
31	European Shorthair	Female	Adult	Misericórdia	No
32	European Shorthair	Male	Juvenile	Santo António	Yes
33	Siamese	Female	Adult	Ajuda	Yes
34	European Shorthair	Female	Adult	Ajuda	No
35	European Shorthair	Female	Adult	Alcântara	No
36	European Shorthair	Female	Adult	Belém	No
37	European Shorthair	Female	Adult	Beato	No
38	European Shorthair	Male	Adult	Carnide	No
39	European Shorthair	Male	Juvenile	São Domingos de Benfica	No
40	European Shorthair	Male	Adult	Ajuda	No
41	European Shorthair	Male	Adult	Santa Maria Maior	No
42	European Shorthair	Male	Adult	Alvalade	No
43	European Shorthair	Female	Adult	Alvalade	No

6. Annexes

Annex 3 (continued).

44	European Shorthair	Female	Adult	Estrela	No
45	European Shorthair	Male	Adult	Alvalade	No
46	European Shorthair	Female	Adult	Alvalade	No
47	European Shorthair	Male	Adult	Alvalade	No
48	European Shorthair	Female	Adult	Alvalade	No
49	Norwegian Forest	Female	Adult	Ajuda	No
50	European Shorthair	Female	Adult	Belem	No
51	Siamese	Female	Adult	Belém	No
52	European Shorthair	Female	Adult	Alvalade	No
53	European Shorthair	Female	Adult	Estrela	No
54	European Shorthair	Male	Adult	Lumiar	No
55	European Shorthair	Female	Adult	Alvalade	No
56	European Shorthair	Female	Adult	Marvila	No
57	European Shorthair	Male	Adult	Marvila	No
58	European Shorthair	Female	Adult	Marvila	No
59	European Shorthair	Female	Adult	Arroios	No
60	European Shorthair	Male	Adult	Alcântara	No
61	European Shorthair	Female	Adult	Marvila	No
62	European Shorthair	Male	Adult	Misericórdia	No
63	European Shorthair	Male	Adult	Santa Clara	No
64	European Shorthair	Male	Adult	Santa Clara	No
65	European Shorthair	Female	Adult	Lumiar	No
66	European Shorthair	Male	Adult	Avenidas Novas	No

6. Annexes

Annex 3 (continued).

67	European Shorthair	Female	Adult	Avenidas Novas	No
68	European Shorthair	Male	Adult	Areiro	No
69	European Shorthair	Male	Adult	Beato	No
70	European Shorthair	Female	Adult	Beato	No
71	European Shorthair	Female	Adult	Benfica	No
72	European Shorthair	Female	Adult	Estrela	No
73	European Shorthair	Male	Adult	Estrela	Yes
74	European Shorthair	Female	Adult	Estrela	No
75	European Shorthair	Male	Juvenile	Santa Maria Maior	No
76	Persian	Male	Adult	São Domingos de Benfica	No
77	European Shorthair	Female	Adult	Misericórdia	Yes
78	European Shorthair	Female	Adult	Alvalade	No
79	European Shorthair	Male	Adult	Alvalade	No
80	European Shorthair	Male	Adult	Alvalade	No
81	European Shorthair	Male	Adult	Areiro	No
82	European Shorthair	Female	Adult	Misericórdia	Yes
83	European Shorthair	Female	Adult	Benfica	No
84	European Shorthair	Female	Adult	Estrela	No
85	European Shorthair	Female	Adult	Misericórdia	No
86	European Shorthair	Female	Adult	Misericórdia	No
87	European Shorthair	Female	Adult	Santa Maria Maior	No
88	European Shorthair	Male	Adult	São Domingos de Benfica	No
89	European Shorthair	Female	Adult	Benfica	No

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Annex 3 (continued).

90	European Shorthair	Male	Adult	Avenidas Novas	No
91	European Shorthair	Male	Adult	Avenidas Novas	No
92	European Shorthair	Male	Adult	Avenidas Novas	No
93	European Shorthair	Female	Adult	Benfica	No
94	European Shorthair	Female	Adult	Avenidas Novas	No
95	European Shorthair	Male	Adult	Belém	No
96	European Shorthair	Female	Adult	Estrela	No
97	European Shorthair	Female	Adult	Arroios	No
98	European Shorthair	Female	Adult	Avenidas Novas	No
99	European Shorthair	Male	Adult	Arroios	No
100	European Shorthair	Male	Adult	Arroios	No
101	European Shorthair	Female	Juvenile	Arroios	No
102	European Shorthair	Female	Adult	Arroios	No
103	European Shorthair	Female	Adult	Avenidas Novas	No
104	European Shorthair	Female	Adult	Chelas	No
105	European Shorthair	Female	Adult	Estrela	No
106	European Shorthair	Male	Juvenile	Chelas	No
107	European Shorthair	Female	Adult	Chelas	No
108	European Shorthair	Male	Adult	Alvalade	No
109	European Shorthair	Female	Adult	Alvalade	No
110	European Shorthair	Male	Adult	Estrela	No
111	European Shorthair	Female	Adult	Alvalade	No
112	European Shorthair	Female	Adult	Alvalade	No

6. Annexes

Annex 3 (continued).

113	European Shorthair	Male	Adult	Campolide	No
114	Siamese	Female	Adult	Avenidas Novas	No
115	European Shorthair	Male	Adult	Avenidas Novas	No
116	European Shorthair	Male	Adult	Avenidas Novas	No
117	European Shorthair	Male	Adult	Lumiar	No
118	European Shorthair	Female	Adult	Alvalade	No
119	European Shorthair	Female	Juvenile	Alvalade	No
120	European Shorthair	Male	Juvenile	Alvalade	No
121	European Shorthair	Male	Adult	Alvalade	No
122	European Shorthair	Male	Adult	Alvalade	No
123	European Shorthair	Female	Adult	Avenidas Novas	No

(WI: without information).

6. Annexes

Annex 4. Sex, age, local of origin and presence of flea infestation of dogs whose blood was donated by the CAL.

Sample	Sex	Age	Local of origin	Flea infested
1	Female	Juvenile	Arroios	No
2	Male	Juvenile	Alcântara	No
3	Male	Juvenile	Alcântara	No
4	Male	Juvenile	Alcântara	No
5	Female	Juvenile	Alcântara	No
6	Female	Juvenile	Santa Maria Maior	No
7	Female	Juvenile	Misericórdia	Yes
8	Female	Juvenile	Alcântara	No
9	Female	Juvenile	Olivais	No
10	Male	Adult	Beato	Yes
11	Male	Juvenile	Chelas	No
12	Male	Adult	Olivais	No
13	Male	Adult	Chelas	No
14	Female	Adult	Unknown	No
15	Female	Adult	Santa Maria Maior	Yes
16	Male	Juvenile	Alcântara	No
17	Female	Juvenile	Olivais	No
18	Male	Adult	Alcântara	No
19	Female	Adult	Santa Clara	Yes
20	Male	Adult	Santo António	No
21	Male	Adult	Lumiar	No
22	No info	No info	NI	No info
23	No info	No info	NI	No info
24	Male	Adult	Benfica	No
25	Male	Adult	São Domingos de Benfica	No

NI – no information provided.