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Estudio molecular de patogenos transmitidos por vectores en perros de Guadelupe (Francia)

Molecular screening of the vector-borne pathogenes circulation in dogs of Guadeloupe (France)

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Molecular screening of the vector-borne pathogens circulating in dogs in Guadeloupe (France)

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ABSTRACT

Keywords: Canine vector-borne diseases, ectoparasites, Caribbean

Vector-borne diseases represent a major health challenge, either because of the complexity of their control, their generally zoonotic nature, or because of the pathology they can cause in the individual. In tropical areas, surveillance of these diseases is even more important, since the presence of vectors is usually continuous throughout the year. In order to develop effective prophylaxis and surveillance programs, it is important to know the identity and prevalence of these pathogens as well as their distribution in a given territory. In Guadeloupe, a French archipelago located in the Lesser Antilles of the Caribbean, although studies have been carried out in ruminants and ticks, no information exists in companion animals. In consequence, blood from forty-six dogs from five different veterinary clinics, located in the two mainland islands, were obtained, and the presence of DNA of the main canine vector borne pathogens (CVBP) by means of diverse PCR protocols. The most frequently detected CVBP was *Coxiella burnetii* (Observed occurrence: 17,39%), followed by *Dirofilaria immitis* (8,70%), and *Candidatus* Mycoplasma haematoparvum, *Hepatozoon canis* and *Rickettsia sp* (2,17% in all cases). All samples were negative for *Anaplasma*, *Ehrlichia*, *Bartonella*, *Borrelia*, *Babesia* and *Leishmania*. No significant differences in pathogen occurrence were observed between the two main islands. This study contributes to fill a relevant gap in the knowledge on vector-borne diseases in the Caribbean.

RESUMEN

Palabras claves: Enfermedades vectoriales caninas, ectoparasitos, Caribe

Las enfermedades transmitidas por vectores representan un importante reto sanitario, ya sea por la complejidad de su control, por su carácter generalmente zoonótico o por la patología que pueden causar en el individuo. En las zonas tropicales, la vigilancia de estas enfermedades es aún más importante, ya que la presencia de vectores suele ser continua durante todo el año. Para desarrollar programas eficaces de profilaxis y vigilancia, es im- portante conocer la identidad y prevalencia de estos patógenos, así como su distribución en un territorio determinado. En Guadalupe, archipiélago francés situado en las Antillas Menores del Caribe, aunque se han realizado estudios en rumiantes y garrapatas, no existe información en animales de compañía. En consecuencia, se obtuvo sangre de cuarenta y seis perros de cinco clínicas veterinarias diferentes, situadas en las dos islas continentales, y se detectaron los principales patógenos caninos transmitidos por vectores (CVBP) mediante diversos protocolos de PCR. El CVBP detectado con mayor frecuencia fue Coxiella burnetii (ocurrencia observada = 17,39%), seguido de Dirofilaria immitis (8,70%), y *Candidatus* Mycoplasma haematoparvum, *Hepatozoon canis* y *Rickettsia* sp. (2,17% en todos los casos). Todas las muestras fueron negativas para *Anaplasma*, *Ehrlichia*, *Bartonella*, *Borrelia*, *Babesia* y *Leishmania*. No se observaron diferencias significativas de presencia entre las dos islas principales. Este estudio contribuye a colmar una importante laguna de conocimiento de las enfermedades transmitidas por vectores en el Caribe.

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INTRODUCTION

1.1 Vector-borne diseases: definition and characteristics

A vector-borne disease (VBD) is an infectious condition transmitted by an organism, known as vector, capable of transmitting the pathogenic agent from one infected individual to another (EFSA, 2024). Vectors are usually hematophagous arthropods, which can transmit infectious diseases. This transmission can be passive or active, i.e. the vector can carry the pathogenic agent and allow it to multiply in its own organism, in which case we speak of a biological vector and the transmission is active (here the vector is also considered as a host), or it can simply transport the agent without multiplication, in which case we speak of a mechanical vector and the transmission is passive.

VBDs are worldwide distributed, affecting humans, domestic animals and wildlife. In humans, they account for 17% of infectious diseases, accounting for more than 700,000 deaths per year (World Health Organization, 2004).

VBDs represent the vast majority of emerging and re-emerging diseases in recent years (Chala & Hamde, 2021). An emerging disease (ED) is an unknown infectious condition that appears for the first time in a given population or area, and a re-emerging disease (RD) is a disease already known that goes on to colonize new regions and/or hosts, or that increases its incidence in endemic areas (World Health Organization, 2004). The greatest concern about these diseases is the immediacy of their impact on health and the economy at the local and international level.

A great challenge of VBDs is to control them effectively. The most widely used method of vector control to date is the use of insecticides, but it poses a risk to human health, the environment and the sustainability of other insects. It also represents a risk for the emergence of vector resistance to these same control methods (van den Berg et al., 2021).

The apparition of EDs or RDs depends on a set of factors such as human activity, the adaptation of microorganisms, the evolution of ecosystems, globalization and climate change. These factors can be summarized by speaking of a rapid evolution of the interfaces between living beings during these last decades, induced in particular by the modification of habitats and movements of individuals (W.-H. Wang et al., 2021). The so-called "spill-over" between species, i.e. the fact that a disease known

to circulate in one species jumps to another species, has been particularly observed in recent years. This jump is considered as emergence of such disease in the new species, and we speak of zoonosis when this transmission involves the human species and another vertebrate (Rahman et al., 2020). Many VBDs are zoonotic, as is the case for West-Nile virus (WNV) disease, Chagas disease, leishmaniosis or Crimean-Congo hemorrhagic fever, which involve companion animals. In particular, dogs are known to be competent reservoirs of many zoonotic agents and are also food sources for hematophagous arthropods (Otranto et al., 2009).

1.2 Major canine vector-borne diseases

Canine vector-borne diseases (CVBD) comprise a relevant and globally distributed group of disease agents (i.e., viruses, bacteria, protozoa, and helminths) affecting wild and domestic canids, transmitted by hematophagous arthropods such as ticks, fleas, lice, triatomines, mosquitoes, and sand flies (Otranto et al., 2009).

In general, the risk of infection by a canine vector-borne pathogen (CVBP) depends on the breed, the animal's genetics, the presence of concomitant infections, its diet, travel between areas with different risks and the animal's environment (community, indoor/outdoor animal) (ESCCAP, 2012). Likewise, as mentioned in the previous section, the probability of infection by a CVBP will depend directly on the abundance of its vector and the environmental conditions of the area (host diversity, human movements, climatic conditions...), and consequently of the management and environment of the dog (deworming, outdoor/ indoor life...) (Di Cataldo et al., 2022).

Canine insect-borne diseases

Leishmaniasis and dirofilariasis are the main canine insect-borne diseases. Canine leishmaniasis is caused by a parasite of the genus *Leishmania*, mainly of the specie *L. infantum* (syn. *L. chagasi*), transmitted by vectors belonging to the *Phlebotomus* genus in Europe, and the *Lutzomyia* genus in the American continent (Ribeiro et al., 2018). Outbreaks are associated with warmer seasons, since the parasite needs temperatures above 18°C to multiply inside the vector and the latter is more active during the hot season (ESCCAP, 2012). The presence of the parasite in the dog's organism can manifest itself in a chronic form with clinical signs for years, in a severe form that can cause the death of the animal, or in an asymptomatic, or latent form, where the parasite infestation enters into equilibrium with the animal's immune system until a possible immunosuppression and appearance of symptoms (Maia & Campino, 2018). As mentioned before, this disease also has a zoonotic character,

having a severe visceral manifestation in humans (Serafim et al., 2020).

Dirofilariosis is also a disease caused by a parasite, a nematode of the genus *Dirofilaria*, transmitted by mosquitoes. Two main types of dirofilariasis are differentiated according to the infecting pathogen: *D. immitis* or *D. repens*, which have a cardiac-pulmonary or subcutaneous manifestation, respectively. Known as heartworm, *D. immitis* can be fatal for the animal if not properly treated, as it lodges in the pulmonary arteries and right ventricle, causing heart failure, edema and thromboembolism. It is mostly distributed in Australia, Southern and Central Europe, South-East Asia and South America (Anvari et al., 2020). Subcutaneous dirofilariasis in turn is zoonotic and is the most prevalent in northen Europe, causing a rarely severe symptomatology in both dogs and humans, generally associated with the appearance of painless cold nodules not adhering to the skin (Joseph et al., 2011).

Apart from these main agents, it is worth mentioning *Bartonella*, which is transmitted by insects, such as fleas, but can also be transmitted by ticks. *Bartonella* is an intracellular bacteria that is adapted to a broad spectrum of mammalian reservoir hosts and is mainly transmitted by (Álvarez-Fernández et al., 2018; Tobar et al., 2020). Domestic dogs can be infected with several Bartonella species or subspecies, including *B. vinsonii subsp. berkhoffii*, *B. henselae*, *B. clarridgeiae*, *B. washoensis*, *B. elizabethae*, *B. quintana*, *B. bovis*, and *B. rochalimae*, and they are known to be reservoirs of *B. vinsonii subsp. berkhoffii* and *B. henselae* (Álvarez-Fernández et al., 2018). *Bartonella* infection in dogs, as in humans, can cause endocarditis and other clinical cardiac symptoms. In the tropics, studies shown *Bartonella* infection in stray and domestic dogs from Colombia, Brazil, Sri-Lanka, and Philippines, with the detection of *B. vinsonii subsp berkhoffii*, *B. rochalimae* and *Candidatus* B. merieuxii (Brenner et al., 2013; Singer et al., 2020).

Canine tick-borne diseases

The main canine tick-borne pathogens (TBP) are babesiosis and hepatozoonosis, which are parasitic diseases, and ehrlichiosis, anaplasmosis and borreliosis, all three of which are caused by bacteria.

The causative agent of babesiosis, which belongs to the so-called piroplasmosis, is a protozoan of the genus *Babesia* that exclusively infects erythrocytes. Most of the *Babesia* spp. are transmitted by *Rhipicephalus sanguineus* sensu lato, while other ticks such as *Haemaphysalis* spp. and *Dermacentor* spp. are more specie-specific (Zygner et al., 2023). In deed, Babesia is generally specific to both the

vector species and the mammalian host, thus each species is distributed according to the presence or absence of its vector. The number of cases of canine babesiosis is apparently increased worldwide in the recent years (Panti-May & Rodríguez-Vivas, 2020). *Babesia* infection can produce from mild signs of apathy and fever to severe hemolytic anemia with periods of recovery and more or less severe relapse, up to lethal, depending on the species involved. Zoonotic potential of this TBP is not proven (Solano-Gallego et al., 2016).

Hepatozoon is another genus of apicomplexan parasites causing hepatozoosis. Unlike *Babesia* spp. and most of the TBPs, transmitted by their haematophageous hosts during a blood meal, *Hepatozoon* spp. transmission takes place by ingestion of the definitive host (ticks, mites, sand flies, tsetse flies, mosquitoes, fleas, lice, bugs, and leeches), and some of them can also be transmitted transplacentally or by ingestion of paratenic host. Infections by the principal specie implicated in dogs, *H. canis*, is associated with anemia, leukocytosis and periostitis as a rare finding. (Baneth, 2011)

Ehrlichiosis and anaplasmosis, are caused by obligate intracellular bacteria of the Rickettsiaceae family. The most important species are *Ehrlichia canis* that infect lymphocytes and monocytes, causing the canine monocytotropic ehrlichiosis, while *Anaplasma platys* infects platelets, causing the thrombocytropic anaplasmosis. Both diseases produce a similar picture of apathy, anorexia, fever and petechiae on the skin and mucous membranes, which is usually accompanied by thrombocytopenia, non-regenerative anemia, hyperglobulinemia and enlarged lymph nodes and spleen (Sainz et al., 2015). Some species are zoonotic, such as *A. phagocyophylum* and *A. platys*, and produce the same pictures in humans as in dogs. It is possible to differentiate between an *Anaplasma* spp. infection and an *Ehrlichia* spp. infection by serological diagnosis or PCR; however, treatment remains the same (Petri, 2024).

Borreliosis are diseases caused by bacteria of the genus Borrelia. The *Borrelia burgdorferi* group, or Lyme Borrelia group, includes 28 species responsible of distinct clinical symptoms, causing the so-called Lyme disease. It is a zoonosis of high prevalence in the United States and Europe that can affect the nervous system of humans in one of its clinical forms. *Borrelia burgdorferi* is transmitted by hard ticks of the genus *Ixodes*, which have a wide range of hosts susceptible to the pathogen, particularly in wildlife (Estrada-Peña et al., 2012).

Beside the major pathogens described previously, it is worth mentioning *Coxiella burnetii*, *Rickettsia* spp. and hemotropic *Mycoplasma* spp, which are rarely related

with severe disease in dogs.

Infections by *C. burnetti* occurs only occasionally in dogs, but exposure increases with the proximity of livestock and ticks (Marrie, 2003). *C. burnetii* is responsible of the Q fever which is a zoonosis that can lead to persistent infections in humans, making it the subject of special monitoring in companion animals in order to prevent possible human outbreaks, as the pathogen has been detected in uterine and vaginal samples in dogs (Abdel-Moein & Zaher, 2021). Infection in dogs is generally asymptomatic but early death of pups has been reported in infected parturient dogs (Marrie, 2003) and outbreaks are associated with parturition and abortion events. Over than 40 species of ticks have been found to carry *C. burnetii*, with the highest prevalence in *Rhipicephalus evertsi* and *A. variegatum* (Yessinou et al., 2022).

The *Rickettsia* genus are pleomorfic obligate intracellular bacteria belonging to the Ricketsiale order. The genus includes zoonotic species of the spotted fever group, such as *R. conorii*, and of the typhus group, such as *R. prowazeki* and *R. typhi* (Walker & Ismail, 2008). These pathogenic Rickettsia are associated with hematophagous arthropods (ticks, mites, fleas, and lice) and affect the endothelial cells of the host (Sahni et al., 2019). Dogs suffer from infection of the mentioned species, being even reservoirs in the case of *R. conorii*, and can express clinical signs of disease although unspecific, such as mild fever, lost of appetite and transient lethargy (Levin et al., 2012; Martínez et al., 2016; Nogueras et al., 2013).

Hemoplasmas are bacteria of the genus Mycoplasma that affect erythrocytes. The two major species infecting dogs are *Mycoplasma haemocanis* and *Candidatus* Mycoplasma haematoparvum, inducing hemolitic anemia only in case of concurrent disease or immunodepression (Tasker, 2022). Poor data are available on the pathogenesis of these species, and their transmission to dogs through ticks is controverted although co-infections with other CVBP is well known (Aquino et al., 2016; Cevidanes et al., 2023; Tasker, 2022). Molecular techniques have confirmed infections in humans with hemoplasma species already reported in dogs and other animal hosts, suggesting that zoonotic transmission is possible (Maggi et al., 2013).

1.3 Focus on the Caribbean

Tropical areas remain the most affected areas by CVBDs (Otranto et al., 2024). As seen above, most of these diseases are specific to their vector and are therefore concentrated in areas where the vector is present. Because of their climatic characteristics and geographical attributes, tropical areas account for more than two-thirds of the world's biodiversity, including insects and ticks as well as a diversity of hosts

involved in the circulation of CVBDs. In addition, climatic changes in these areas cause a net geographic expansion of CVBDs in recent years, and warmer temperatures have indeed been shown to affect the behavior, physiological characteristics and life cycle of vectors and pathogens (Thomson & Stanberry, 2022).

The Caribbean is a cosmopolitan area, at the crossroads of intercontinental exchanges between the Americas, Europe and Africa, which particularly encourages the introduction and spread of vectors and VBDs, especially through the movement of animals (through legal or illegal trade and bird migration). Despite the high prevalence of animal and zoonotic infectious diseases in this area, limited epidemiological knowledge of the diversity of vectors and vector-borne pathogens circulating there, together with the lack of experience in the management of VBDs, limit the development of effective control strategies (Gondard et al., 2020).

Information about the epidemiological situation of CVBDs in the Caribbean area is scarce and limited to a handful of islands. Studies carried out in dogs, in Haiti, Grenada, Trinidad, St. Kitts, Cuba and the Dominican Republic, showed high prevalences in dogs of Anaplasma (mainly *A. platys*), Babesia (*B. vogeli* and *B. gibsoni*), *H. canis* and *D. immitis*; other vector-borne pathogens have been found at a lower prevalence, such as Bartonella (*B. haensalae* and *B. vinsonii*) and Hemoplasma (*Mycoplasma haemocanis* and haematoparvum) (Georges et al., 2008; Roblejo-Arias et al., 2022; Yabsley et al., 2008). *Borrelia burgdorferi* has never been reported in the Caribbean (Maggi & Krämer, 2019; Starkey et al., 2016).

1.4 The particular case of Guadeloupe

The archipelago of Guadeloupe (2.1), is a French territory located in the Caribbean. The Guadeloupean archipelago concentrates a great diversity of terrestrial and marine ecosystems, making it, like the rest of the Caribbean, one of the 34 biodiversity hotspots in the world (Maunder et al., 2008). Guadeloupe also has many connections, both touristic and commercial, with neighboring Caribbean countries and territories, North America and Europe. These large flows of people, animals, plants and by-products favor the spread of emerging pests and diseases. Thus, in the past decades, the archipelago has suffered several outbreaks of emerging infectious diseases in both humans and animals (West Nile virus, Chikungunya, dengue, leptospirosis, etc.) (Gruel et al., 2021).

Since the detection in the 1980s of the circulation of *Ehrlichia ruminantium*, transmitted by *A. variegatum* ticks and responsible for ruminant hydrocarditis (cowdriosis), several studies on VBD in ruminants, particularly those transmitted by ticks,

have been initiated in the archipelago. Sampling of ticks feeding on ruminants has demonstrated the high prevalence of *A. variegatum* and *Rhipicephalus* (*Boophilus*) *microplus* in the archipelago (Camus & Barre, 1995). *Amblyomma variegatum* is a tropical tick widely distributed throughout the Caribbean and requires 3 hosts for its life cycle. In Guadeloupe, this species is recognized to be responsible for the transmission of *Ehrlichia ruminantum*, *Theileria mutans*, *Theileria parva*, *Rickettsia africae* and *Rickettsia conorii* in ruminants. *R. microplus* in turn, is considered worldwide to be the bovine tick. It feeds on a single host throughout its life cycle, and in the archipelago is capable of transmitting *Anaplasma marginale*, *Babesia bigemina* and *Babesia bovis* to ruminants. Another tick species, such as *Dermacentor nitens*, responsible for the transmission of *Babesia cabali* in goats, sheep and equids, has been detected in the archipelago. (Gondard et al., 2017)

As for arthropods, about 40 different species of mosquitoes has been identified in Guadeloupe (ARS Guadeloupe, 2018). WNV surveillance studies conducted over the Guadeloupean mainland have provided information regarding the abundance of the species possibly implicated in the transmition of the virus: *Culex atratus*, *Culex nigripalpus*, *Deinocerites magnus* and *Ochlerotatus taeniorhycus* (Geffroy et al., 2021; Imbert, 2018; Pradel et al., 2009), all being able to feed on warm-blooded animals, such as mammals and birds. Apart from these species, the most abundant in the urban areas of the archipelago, and responsible for the transmission of many emerging and re-emerging arbovirosis such as Dengue or Chikungunia, is *Aedes aegypsi* (ARS Guadeloupe, 2018; Yen et al., 2018).

Other arthropods of little interest epidemiologically, are also monitored for possible outbreaks of diseases of special public health concern. This is the case of mosquitoes of the genus *Anopheles*, capable of transmitting malaria, which are part of a special surveillance although no autochthonous case of the disease has been demonstrated in Guadeloupe (Ramdini et al., 2012).

Phlebotomine sandflies, known to be responsible for the transmission of leshmaniosis, are present in the archipelago but the species found are not competent for the transmission of the parasite (Ramdini et al., 2012). However, a case of leishmaniosis due to *L. martiniquensis* has been detected in a human in Martinique (Liautaud et al., 2014). Findings of *Leishmania* spp. has also been made in *R. microplus* ticks collected on cattle from Martinique (Gondard et al., 2020). These findings are of special interest in Guadeloupe because both islands are socially and economically connected, with daily exchanges of people, goods, and animals.

1.5 Project objectives

All the work mentioned above was mainly focused on identifying the vectors present in the archipelago and studying their ecology, in order to improve vector control strategies and optimize the control of vector-borne pathogens affecting production animals or humans (Gondard et al., 2017, 2020). However, no information is available on the CVBDs circulating in Guadeloupe, nor on the vectors involved in their transmission. Veterinarians have been able to diagnose certain CVBDs in dogs, such as ehrlichiosis and dirofilariosis, based on symptomatology and/or diagnostic tests (unpublished data), but no molecular surveys have been performed to date to confirm those observations. Knowing the exact etiology of the CVBPs in a given area is primordial to carry out accurate diagnosis and treatments, and to establish appropriate control measures.

This work aims to provide for the first time molecular and epidemiological data on the CVBPs circulating in domestic dogs in the French archipelago of Guadeloupe, and to assess potential intrinsic and extrinsic factors related to their presence.

Chapter 2

METHODS

2.1 Study area

The study was carried out in the Guadeloupean archipelago, in the Caribbean region, which is located between the Tropics of Cancer and Capricorn, southeast of the Gulf of Mexico, east of Central America and north of South America. It consists of more than 700 islands, islets, reefs and cays, bounding the Caribbean Sea by two major island arcs: the Greater Antilles to the north, and the Lesser Antilles to the south and east (2.1).

During most of the year, temperatures hover around 30°C and most of the rainy days are concentrated from May to October, with an average of 70 to 178 mm/month, which gives it its tropical climate.

Figure 2.1: Geographical location of the Caribbean. Guadeloupe is indicated by the red circle. Modified from (Thomson & Stanberry, 2022)

Being part of the Caribbean region, Guadeloupe average annual temperature rounds the 27°C and its relative humidity the 80% (wofrance.fr, n.d.). Fluctuations in temperature and humidity vary especially between the dry season, from January to June, and the rainy season, from July to December. The archipelago can be divided in:

- A "continental" part, composed of the two major islands, Grande-Terre (GT)

and Basse-Terre (BT), closely connected (as they are only separated by a narrow arm of the sea)

- A group of annexed islands, including the small archipelago of Les Saintes in the south-west, the island of Marie-Galante in the south, and the island of Désirade in the east.

2.2 Animal Sampling

The type of methodological approach was cross-sectional. Veterinary clinicians were approached and requested to take a sample during their practice. Clinics were chosen trying to cover the entire territory of the continental part of the archipelago. Five clinics agreed to participate in the project: three from the GT island and two from the BT island (2.2).

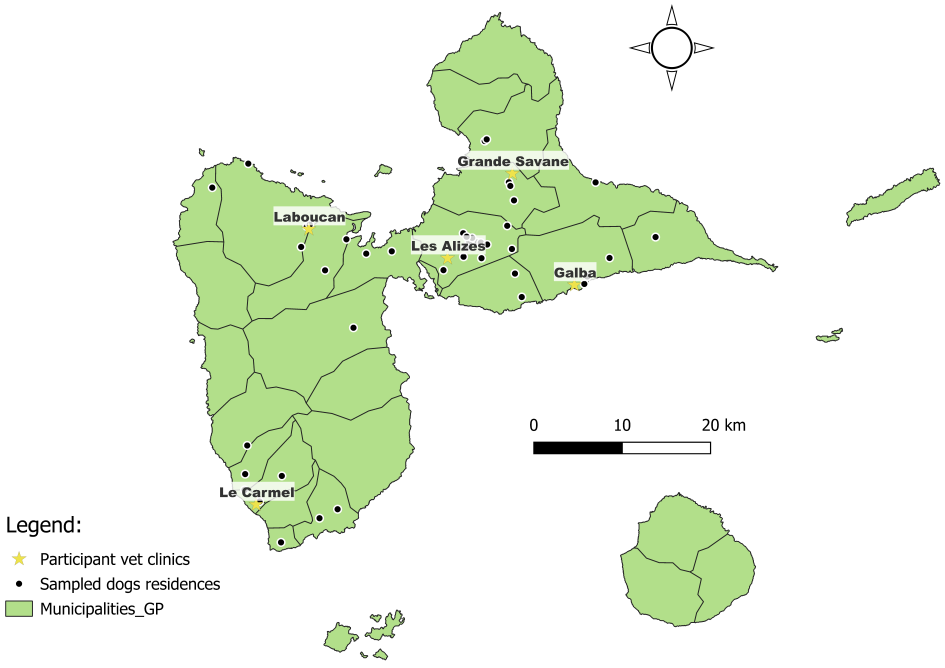

Figure 2.2: Guadeloupe archipielago with the origin of the sampled dogs and name of the participant veterinary clinics. GT is the eastern island and BT is the western one.

Between the June 15th-30th of 2023, blood samples were collected from the jugular or the cephalic veins of dogs. A drop (about 100 uL) was applied to a FTA™ Nucleic Acid Collection Cards (Whatman, Maidstone, Kent, UK), air-dried and placed into

Eppendorf tubes. These filter papers ensure a long-term storage, stabilization and protection of nucleic acids at room temperature.

For each sample, the following information related to the patient was registered: age, breed, sex, place of residence, parasitic treatments, vaccines, previous clinical history, and presence of ectoparasites (A.1).

2.3 Sample size calculation

Population size: According to (Gompper, 2014), the domestic dog population rounded 115,800 individuals in Guadeloupe in 2011. Taking the actual data of the whole French territory from the annual report of (FEDIAF, 2022), and relating it to the human population size of the guadeloupean archipelago (INSEE, 2022), the domestic dog population can be estimated around 33,430 in 2023. Thus, the population size range taken for the estimations were [33,430; 115,800]. In any case, both estimations are above 10 000, which does not affect the calculation.

Expected prevalence: Among the pathogens studied in this work and according to the literature, *D. immitis* was the CVBP encountered at higher prevalences in the Caribbean (mean: 20,8% (Chikweto et al., 2014; Starkey et al., 2016)). Taken this population size and such expected prevalence as a reference, with a 90% confidence level and a precision of 5%, in the studied population, the sample size required should have been 179 individuals according to WinEpi (A.2). Unfortunately, only 46 samples could finally be obtained.

2.4 DNA extraction

A circular punch of 1mm (diameter) of the blood spot was placed in a microtube for the DNA extraction process.

A first cleaning was done with 180µl of Tris-EDTA in order to remove preservative substance and possible PCR inhibitos from the sample. After a short spin and 15 minutes, all the Tris-EDTA was removed and replaced by 180µl of extraction medium. The extraction medium consisted of an aqueous solution of Chelex® sigma resin (5% w/v). Tubes were then quickly vortexed and placed in a thermoclycler for 10 minutes, at 100°C. After the program ended, the tubes were vortexed another time and submitted to a second 100°C incubation for 25 minutes. In the end, the total volume of DNA extraction medium were 180µl for each sample.

In order to verify the good DNA extraction of each sample, a verification PCR has been carried out for the gene MC1R (coding for the melanocortin1 receptor),

present in all canine genomes. For a total PCR mix of 10µl, 5µl of DNA sample were added to:

- 1µl of each primer (forward and reverse),
- 1µl of incomplete buffer x10 (doesn't include Mg),
- 0,3µl of Mg (50 mM),
- 0,8µl of dNTPs (2,5 mM each),
- 0,06µl of Taq Polymerase from IBIAN™ (5 U/µl)
- 0,84µl of distilled water.

The PCR protocol used is described in (Tab 2.1). PCR products were reveled by electrophesis on a 1% agar gel.

Whenever DNA extraction did not provide a successful result, a new punch was obtained and submitted to two Tris-EDTA washes prior to Chelex incubations, in order to decrease the concentration of possible contamination that inhibits the DNA. This way, DNA from all the 46 samples was obtained.

2.5 Pathogen detection

A selection of the main CVBP was made, based on the literature and considering the findings made in other places of the Caribbean and South-America. The presence of DNA of pathogens belonging to the following genus was tested: *Anaplasma*, *Ehrlichia*, *Rickettsia*, *Leishmania*, *Bartonella*, *Borrelia*, *Coxiella*, hemotropic *Mycoplasma*, *Babesia* and *Hepatozoon*; and the family Filariidae.

From the 46 DNA extracts, presence of DNA of the different pathogens was tested by PCR, using the primers and protocols shown in (Tab 2.1). All PCR were run with a DNA proportion of 40% of the final PCR mix, using the Promega™ PCR Master Mix (5µl of Master Mix, 0,5µl of each primer and 4µl of DNA). For certain genus, multiple genes were targeted in order to improve the probability of having a correct result. Part of the PCR product was reveled by electrophesis on a 1% agar gel.

A first screening PCR was carried out to detect both Piroplasmas and *Hepatozoon* spp. Secondly, the positive samples were discriminated by specific PCR for *Babesia* sp. and *Hepatozoon* sp.

Target gene	Primers F/R	Initial denaturation	Denaturation	Annealing	Extension	Number of cycle	Fragment length (bp)
MC1R	ChocF/ChocR	94°C, 5'	94°C, 30"	60,2°C, 30"	72°C, 30"	35	450
Anaplasma spp. (16S)	16SF/16SR	94°C, 2'	94°C, 30"	42°C, 30"	72°C, 1'	35	421
Anaplasma spp. (RpoB)	RpoBF/RpoBR	95°C, 2'	95°C, 30"	55°C, 45"	72°C, 45"	35	577
Anaplasma spp. (Glta)	F1b/HG1085R	95°C, 2'	94°C, 30"	45°C, 45"	72°C, 45"	35	459
Ehrlichia spp. (16S)	16sR/16sD	94°C, 2'	94°C, 30"	62°C, 30"	72°C, 30"	35	345
Rickettsia spp. (16S)	fd1/Rc16	94°C, 1'	94°C, 30"	54°C, 30"	72°C, 1'	40	416
Rickettsia spp. (OmpA)	OmpAF/OmpAR	94°C, 1'	94°C, 30"	54°C, 30"	72°C, 1'	35	650
Rickettsia spp. (OmpB)	OmpBF/OmpBR	94°C, 1'	94°C, 30"	53°C, 30"	72°C, 30"	35	618
Rickettsia spp. (Glta)	GltAF/GltaR	95°C, 2'	94°C, 30"	45°C, 45"	72°C, 45"	35	360
Mycoplasma spp. (16S)	Mycop16S rRNA-F/Mycop16S rRNA-R	95°C, 4'	95°C, 15"	60°C, 30"	72°C, 30"	40	384
Mycoplasma hemocanis (16S)	Mhf-OH-OK1/Mycop-00CB-r1	95°C, 5'	95°C, 45"	58,4°C, 45"	72°C, 30"	35	175
Candidatus M. haematoparvum (16S)	M sp/C Mhp	95°C, 4'	95°C, 30"	56,5°C, 30"	72°C, 20"	35	175
Bartonella spp. (ITS1)	ITS1F/ITS1R	95°C, 2'	95°C, 30"	59°C, 45"	72°C, 45"	39	675
Bartonella spp. (Glta)	BhCS871.p/BhCS1137.n	95°C, 2'	95°C, 30"	51°C, 45"	72°C, 45"	39	321
Bartonella spp. (RpoB)	rpoB 1400F Barto/ rpoB 2300R Barto	95°C, 2'	95°C, 30"	53°C, 45"	72°C, 1'	39	379
Borrelia spp. (23SN1/2)	23SN1/23SC1	95°C, 1'	94°C, 30"	52°C, 30"	72°C, 45"	35	380
Nested PCR*	23SN2/5SCB	95°C, 1'	94°C, 20"	52°C, 20"	72°C, 55"	35	225
Coxiella burnetii (IS1111) Conventional PCR	Trans B/Trans M	94°C, 5'	94°C, 30"	63°C, 1'	72°C, 1'	35	337
Coxiella burnetii (IS1111) qPCR	QKF3/QKR3	95°C, 10'	95°C, 15"	60°C, 1'		50	78
Piroplasmas/ *Hepatozoon* (18S)	PiroA/PiroB	95°C, 5'	95°C, 30"	64°C, 45"	72°C, 30"	34	360
Babesia spp. (18S)	PiroF/PiroR	95°C, 5'	95°C, 30"	58°C, 30"	72°C, 30"	35	408
Hepatozoon spp. (18S)	HepaF/HepaR	95°C, 5'	95°C, 30"	50°C, 30"	72°C, 30"	35	574
Leishmania spp. (ITS)	ITSR/L58S	95°C, 5'	95°C, 30"	52°C, 45"	72°C, 45"	40	720
Filariidae (ITS2)	DIDRF/DIDRR	95°C, 2'	95°C, 30"	60°C, 30"	72°C, 30"	35	302 (*D. immitis*)
Filariidae (COI)	COIfilF/COIfilR	94°C, 5'	95°C, 30"	52°C, 45"	72°C, 45"	35	631

Table 2.1: PCR protocols. ' = minutes, " = seconds, F = Forward, R = Reverse. *Nested PCR consists in running 2 PCR. The second being run with the PCR product of the first, amplifying a shorter fragment by using another set of primers. The objective is to reduce non-specific bindings and increase the sensitivity of the PCR.

For the detection of *Coxiella burnetii*, a quantitative PCR (qPCR) was carried out, in addition to the conventional one. qPCR enables a computerized monitoring of the DNA amplification, allowing to know exactly how many PCR cycles (cycle threshold: Ct) have been run before reaching the threshold of positivity. This method is relevant when the DNA concentration present in the sample is low. In this case, Nanodrop quantification proved a very small concentration of DNA in every sample (average of 3,3 ng/µl).

2.6 Sequencing

Amplicons obtained from positive PCR protocols were sent for sequencing to MacroGen (Madrid, Spain). When a PCR product revealed multiple or poorly-defined bands on the electrophoresis gel, the gel was cut at the weight corresponding to the desired sequence, and extracted. In the other cases, the PCR product left in the microtube was directly used.

The primary genomic sequences obtained were read and analyzed with Chromas™ software. They were then pasted in the Blast program of the NCBI™ genomics database, to identify possible correspondences with a known specie.

2.7 Statistical and epidemiological analysis

Sample size calculations, confidence intervals (CI) of the observed occurrences and maximum possible prevalences were calculated with the Winepi™ program.

Fisher's exact tests were run to analyse the homogeneity of the observed frequencies between the two islands, using R™ program.

Maps representing the environmental and clinical information of the sampled individuals were made with QGIS™ software. *Shapefile* layers were downloaded from the *KaruGéo*™ portal for the wetlands, the Geonetwork™ opensource for the municipalities, A. Cousteau archives for the ecosystems, and self-made with QGIS™ for the specific information of this study.

Chapter 3

RESULTS

3.1 Pathogen detection and observed occurrences

At least one pathogen was detected in 30% of the dogs. Out of the 46 blood samples, eight were found positive to *C. burnetii*, four to Filariidae, one to *Ricketsia* sp., one to *Hepatozoon sp* and one to *Mycoplasma* sp. (Tab 3.1).

The eight samples positive to *C. burnetii* with qPCR, with a mean of 39,45 Ct, were negative with conventional PCR. Due to the small sequence length, sequencing were not conclusive for any of the eight samples.

The four Filariidae-positive samples, were subjected to a second PCR protocol for molecular characterization (D.4) (D.5). All these sequences showed 100% identity with *D. immitis* sequences (GenBank accession number: MK250756) (D.6) (D.7)(D.8)(D.9).

Two samples were found positive with the screening PCR for Piroplasmas and *Hepatozoon* (Tab 3.1) (C.1). After running the specific PCR for *Babesia* spp. and *Hepatozoon* spp. in both samples, only one was positive for *Hepatozoon* spp., none for *Babesia* spp. (C.2) (C.3). Sequencing of the positive case of Hepatozoon yielded sequences 99% similar among them, with an identity of 99,81% with published sequences of *H. canis* (accession number: MT081050) (D.1).

Sequencing of *Mycoplasma* amplicons showed 99,69% homology with *Candidatus* M. haematoparvum (accession number: KY117661) (D.2).

Sequencing of the *Rickettsia* positive sample showed an identity of 96,76%,with uncultured *Rickettsia* spp. (Genbank accession number: KT733038) and with several fragments of *Rickettsia aeschlimannii* (Accession numbers: KY233291, KY233290, MF098413.1, MW398879, MW398877)(D.3). Unfortunately, none of the additional protocols for Rickettsia turned out positive, preventing us to characterize the case.

No dog was positive for *Anaplasma*, *Ehrlichia*, *Bartonella*, *Borrelia*, *Babesia* and *Leishmania*. For these pathogens, the maximum possible prevalence according to WinEpi was 6.3% (B.1).

Only one dog was co-infected, in that case by *Candidatus* M. haematoparvum and *D. immitis*.

Detected pathogen	Observed occurrence (%)	95% CI
Coxiella burnetii	17.39	[6.45 ; 22.34]
Dirofilaria immitis	8.70	[0.56 ; 16.83]
Candidatus M. haematoparvum	2,17	[0.00 ; 6.39]
Hepatozoon canis	2.17	[0.00 ; 6.39]
Rickettsia spp.	2.17	[0.00 ; 6.39]
Overall	30.43	[17.14 ; 43.73]

Table 3.1: Observed occurrences with confidence intervals. Overall: proportion of individuals infected with at least one pathogen

3.2 Clinical and epidemiological data

Coxiella burnetii-positive dogs were found all over the continental territory without notable aggregation in a specific area or ecosystem (3.1). Moreover, no significant difference between islands (3 from 27 in GT, 5 from 19 in BT) were found (Fisher's p-value = 0.2455) (B.2). From the eight positive dogs, three were treated with only internal anti-parasitic treatments, two with only external anti-parasitic treatments, 1 with both and two without anti-parasitic treatment. No clinical history compatible with infection with Coxiella was reported from any of these dogs.

The four positive dogs to *D. immitis* were from the center and North of the GT island, mainly in the region of the "Grands-fonds" (3.1) (yellow and red dots). As said, one of these individuals were co-infected with *Candidatus* M. haematoparvum (red dot). From the four dogs, one was already diagnosed with heartworm disease by antigenic test, and the co-infected dog was noticed with hematuria and coughs during the harvesting period. No relevant clinical history was reported for the two remaining positive dogs. None of those was regularly treated against parasites. Historically, *D. immitis* was mainly diagnosed by the Guadeloupean veterinarians in the North of the GT island, but during recent years it seems to show a clear geographical expansion. However, frequencies obtained in the two islands (4 from 27 in GT, 0 from 19 in BT) are not significantly different (Fisher's p-value = 0,1313) (B.3).

Positive dogs to *Rickettsia* sp. and *H. canis* were respectively from the North-East and South of BT Island (3.1) (purple and blue dots, respectively). None has clinical history or clinical symptoms. The Hepatozoon-positive dog received only treatment for external parasites.

Regarding the overall positive dogs of the study, no significant difference between islands (7 from 27 in GT, 7 from 19 in BT) have been demonstrated (Fisher's p-value = 0.5217) (B.4).

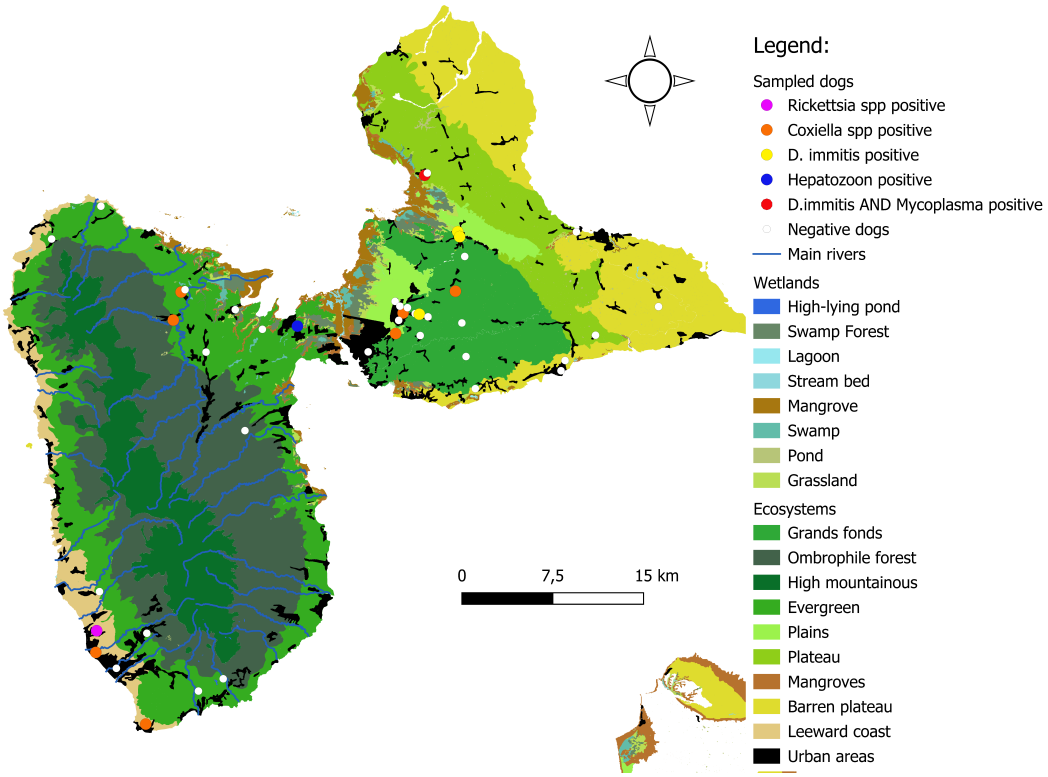

Figure 3.1: Pathogens distribution in the study area.

Chapter 4

DISCUSSION

The present work represents the first study of CVBP in Guadeloupe, a Caribbean island highly connected with the rest of the Caribbean region, Europe and the American continent. Indeed, pathogens transmission is favoured by animal shipping from a country to another. Thus, identifying the CVBP present in the archipelago is key to understand the dynamic of such pathogens among the Caribbean, control them and prevent outbreaks. Notwithstanding, we observed a low pathogen variability among the sampled dogs and only one case of co-infection, which is generally observed in the Lesser Antilles but differs with larger islands, or close to the continent, and continental territories (Georges et al., 2008; Kelly et al., 2013; Loftis et al., 2013; Maggi & Krämer, 2019; Starkey et al., 2016; Yabsley et al., 2008). This phenomenon can be associated with the negative diversity-isolation of the "island effect", whereby continental territories and nearby islands are sources of biodiversity for the little islands, decreasing as we move away from the continent (D. Wang et al., 2022). On the other hand, Guadeloupean veterinarians generally affirm that TBD, such as ehrlichiosis, anaplasmosis, babesiosis and hepatozoosis, are generally less diagnosed in the past 20 years, associated with the intensive parasite control campaigns in ruminants, especially in GT island, probably leading to a global decrease in ticks abundance, and consequently in TBD prevalence. However, we cannot totally rule out the presence of some of the undetected pathogens, given the small sample size of the study. In addition, the type of sample used (a blood spot in a filter paper), may affect the quality of the extracted DNA.

In the Caribbean *Coxiella* spp., specifically *C. burnetii*, is mostly found in cattle, sheep and goats. Nevertheless, the bacteria has been identified in ticks collected on horses in Cuba (Noda et al., 2016), and one cat has been reported with positive serum in St Kitts (Johnson et al., 2020), but no report of infected dogs has been done until now. More, a study carried out among military dogs in Martinique, showed no evidence of Coxiella infection while dogs from the other French territory studied showed positivity (Boni et al., 1998), especially in French Guyana (Boni et al., 1998; Epelboin et al., 2023). Therefore, the present work is the first report of *C. burnetii* infections in dogs in the Caribbean. Although *C. burnetii* infections in dog is generally asymptomatic, the zoonotic nature of this CVBP represents a public health concern. Surveys should be carried out over all the archipelago in

domestic animals, especially those in contact with ruminants and milk tanks, in order to prevent possible outbreaks in humans.

All the Filariidae-positive samples were identified as *D. immitis*. In the Caribbean *D. immitis* is very frequent in dogs (Maggi & Krämer, 2019; Starkey et al., 2016), and seems to be expanding in the archipelago from the North of GT in the last decades, according to the local veterinarians (pers. comm.). Mosquito bites are almost inevitable due to the annual climatic conditions of the archipelago, thus, the use of mcyclic lactones (ML) against *D. immitis* larva is the most popular prevention method. However, apparition of ML-resistant *D.immitis* strains calls for prevention strategies (Prichard, 2021). Studies showed that the initial apparition of ML resistance happened in an area with intensive treatment and high transmission, with minimal influx of new mosquitos (Geary, 2023), which could be likened to island conditions. Although the present work couldn't show a clear difference of occurrence between the two main islands, it is interesting to highlight that the two islands have different geoclimatic characteristics: while GT is a limestone island with dry forest, plains and barren plateau, BT is a volcanic islands with ombrophile forest and high mountains, creating different micro-climates where the abundance of the different mosquito species would be interesting to investigate. Further *D.immitis* surveys with a larger sample size should be carried out to identify the more susceptible zones, and consequently limit the use of ML as a preventative to the higher risk areas. *Hepatozoon canis* is rarely found in Latin-America, although high prevalence has been found in some rural areas of Brazil and Costa-Rica, and it is widely spread in the Caribbean islands (Kelly et al., 2013; Loftis et al., 2013; Maggi & Krämer, 2019; Starkey et al., 2016; Yabsley et al., 2008). The observed occurrence in this work (2,17%) is lower than the mean prevalence reported in the rest of the Caribbean (around 9%) (Kelly et al., 2013; Loftis et al., 2013; Starkey et al., 2016; Yabsley et al., 2008).

Regarding *Rickettsia* sp., only one positive sample was found and we were not able to fully characterize it. The potentially identified species, *Rickettsia aeschlimannii*, which belongs to the Rickettsia spotted-fever group (SFG), has been reported in infected humans but never in other mammals, although it has been found in ticks collected on small ruminants, domestic dogs, and cattle in Pakistan(Majid et al., 2023). *Rickettsia aeschlimannii* has never been reported in the Caribbean, although other species from the SFG, such as *Rickettsia africae* and *Rickettsia conorii* has been found in dogs (Kelly et al., 2013; Maggi & Krämer, 2019). In Guadeloupe, *Rickettsia africae* and *Rickettsia conorii* has been reported in humans, cattle and goats, but no other Rickettsia spiece has ever been reported (Gondard et al., 2020;

Parola et al., 1999).

In the Caribbean *Candidatus* M. haematoparvum was reported in dogs from Cuba (Roblejo-Arias et al., 2022), and in a woman from Grenada (Maggi et al., 2013). The other canine hemoplasma, *M. haemocanis*, was not found in our sampling. This can be the result of true absence due to the island effect abovementioned, or the consequence of failing to reach the required sample size. Transmission routes of the canine hemoplasmic Mycoplasmas are not well known. Altough originally these were considered to be transmitted by ticks, other ways of transmission, such as via predation, fights or vertically, cannot be excluded (Cevidanes et al., 2023; Maggi et al., 2013; Tasker, 2022). This incognita represents an obstacle to the sustainable development of effective control strategies. In Guadeloupe, the tick species suspected to be able to transmit hemoplasmas, such as *R. sanguineus* sensu lato, are present (Gondard et al., 2020; Tasker, 2022). Combined with the presence of feral dogs all over the territory, it should represent a great concern for the transmission of the pathogen in the archipelago, keeping in mind its zoonotic potential (Maggi et al., 2013).

Chapter 5

CONCLUSIONS

5.1 English version

In conclusion, this project is a first step to encourage more extensive studies on the CVBP circulating in Guadeloupe. Although the provided data are based on a limited sampling, I was able to confirm several observations made by local veterinarians and identify by molecular means, for the first time, the presence of relevent CVBP on the Guadeloupean territory, some of them with zoonotic potential. All these pathogens were previously confirmed in other Caribbean islands, except for *C. burnetii* which is reported for the first time in this study. This clears the way for further studies to determine the actual prevalence of the detected CVBP and their vectors, to implement surveillance campaigns and design efficient prevention measures in Guadeloupe.

5.2 Versión en español

En conclusión, este proyecto es un primer paso para fomentar estudios más amplios sobre los patógenos caninos transmitidos por vectores que circulan en Guadalupe. Aunque los datos proporcionados se basan en un muestreo limitado, pude confirmar varias observaciones realizadas por veterinarios locales e identificar por medios moleculares, por primera vez, la presencia de patógenos relevantes en el territorio guadalupeño, algunos de los cuales tienen potencial zoonóitico. Todos estos agentes patógenos habían sido confirmados anteriormente en perros de otras islas del Caribe, a la excepción de *C. burnetii* que se señala por primera vez en el Caribe en este estudio. Deberán realizarse nuevos estudios para determinar la prevalencia real de los patógenos detectados y sus vectores, a fin de poner en marcha campañas de vigilancia y medidas de prevención eficaces en Guadalupe.

Chapter 6

PERSONAL ASSESSMENT

During the realization of this project I have been able to connect again with the Guadeloupean animal health problematics and interact with different veterinary practitioners of my native land, which is very important for me on both personal an professional aspects. Being able to add knowledge as a local actor is a privilege that I am thankful for. Moreover, I was able to reuse and reinforce knowledge and skills I had acquired during my previous studies, in human health and epidemiology, added to all that I have been able to learn on the different CVBD and the situation in the Caribbean and Latin American region. I also had the big chance to be helped by investigators from different structures (IA2, IREC and the Genetic department of the veterinary faculty of Zaragoza), which enriched my knowledge and gave me the opportunity to increase my scientific rigour.

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