ESCOLA UNIVERSITÁRIA VASCO DA GAMA

MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

Coxiella burnetii in small animals: Serological and molecular study in female dogs

Samuel Costa Anjos

Coimbra, julho 2021

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For Nina

“In the whole history of the world, there is but one thing that money cannot buy...to wit the wag of a dog's tail.”

— Josh Billings

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List publications

- **Comunication accepted for presentation in the annual congress of *European College of Veterinary Internal Medicine* (ECVIM), 2021**

Serosurvey of *Coxiella burnetii* in companion animals from Portugal (Annex VIII)

Samuel Anjos, Suzi Neves, Tiago Neves, Pedro Esteves, Hélder Craveiro, Maria dos Anjos Pires, Hugo Vilhena, Sofia Anastácio

- **Communication submitted in IMED (International Meeting on Emerging Diseases and Surveillance), 2021**

Serosurvey of SARS-CoV-2 in dogs and cats from Portugal (Annex VII)

Suzi Neves, Samuel Anjos, Tiago Neves, Pedro Esteves, Hélder Craveiro, Bruno Medeira, Hugo Vilhena, Sofia Anastácio

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± - More or less

^oC – Degrees Celsius

% - Percent

µL – Microliter

CFT - Complement Fixation Test

CI – Confidence interval

Ct - Cycle threshold

DNA - Deoxyribonucleic acid

EDTA - Ethylenediaminetetraacetic acid

e.g. – For example

ELISA – Enzyme-Linked Immunosorbent Assay

GA, USA – Georgia, United States of America

IFA - Immunofluorescent assay

IgG – Immunoglobulin G

IgM – Immunoglobulin M

LCV - Large-cell variants

mg – Milligram

mL - Milliliter

n.a. – Not applicate

NC - Negative Control

nm – Nanometer

Nr – Number

OD - Optic Density

OIE – World Organization for Animal Health

PBS - Phosphate Buffered Saline

PC - Positive Control

PCR - Polymerase Chain Reaction

Q fever – Query fever

qPCR – Quantitative Polymerase Chain Reaction

SCV - Small-cell variants

SD - Standard deviation

SDC - Small dense cells

S/P – Sample to positive ratio - optic density of the sample / optic density of the positive control sample

Taq Polymerase - thermostable DNA polymerase

Coxiella burnetii in small animals: serological and molecular study in female dogs

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Resumo

Coxiella burnetii é o agente causador da zoonose Febre Q. Esta apresenta uma ocorrência mundial e os ruminantes domésticos são os hospedeiros mais comuns de *C. burnetii* e fonte da infeção humana. Os cães podem também ser infetados, contudo, o seu papel na epidemiologia desta bactéria ainda não está claro.

Este estudo foi realizado em cadelas pacientes de centros de atendimento médico-veterinário das regiões norte e centro de Portugal, entre outubro de 2020 e março de 2021, para realização de ovariohisterectomia. Amostras de sangue/soro/plasma excedentes de análises de rotina e tecidos excedentes de procedimentos cirúrgicos foram colhidas e utilizadas para a realização dos testes serológicos e moleculares. Durante o período de estudo foram sujeitos a amostragem um total de 78 animais diferentes (n=78). As amostras de soro/plasma foram testadas para detetar a presença de anticorpos específicos anti-*C. burnetii* recorrendo a um teste ELISA comercial adaptado para várias espécies (ID Screen Q Fever Indirect Multispecies®, IDVet®).

Na pesquisa de anticorpos, os resultados foram expressos em valores S/P%. Um S/P% > 50% foi classificado como positivo. Um total de sessenta (n = 60) animais foram testados para anticorpos anti-*Coxiella burnetii*. A amostra foi composta por trinta cães de raça pura e trinta animais de raça cruzados, com uma variação de idades entre cinco meses e quinze anos. Apenas um resultado positivo (S/P%= 54.96) foi detetado correspondendo a uma cadela de seis anos residente numa zona rural. A taxa de seropositividade foi assim determinada em 1,7% (CI 95%: 0,3 a 9,1%). Um resultado duvidoso (S/P% = 43%) foi detetado numa outra cadela de cinco anos residente numa zona semirrural.

Para a deteção DNA de *C. burnetii* no tecido reprodutivo, foram realizados testes de qPCR em 67 amostras.Todas as amostras apresentaram um resultado negativo, não tendo sido evidenciada a presença de *C. burnetii* nas amostras testadas.

A taxa de exposição encontrada nos animais testados foi muito baixa nos testes serológicos. Isto pode sugerir que as cadelas das regiões norte e centro de Portugal não são frequentemente expostas à bactéria ou por outro lado a amostragem de conveniência pode não ser representativa da população comprometendo as conclusões por enviesamento dos resultados.

No entanto, o estudo e a vigilância da infeção por *C. burnetii* em animais de companhia podem ser uma mais-valia para prevenir surtos e a transmissão para tutores e veterinários em contato com animais infetados.

Palavras-chave: febre Q, animais de companhia, ELISA, qPCR, zoonose

Abstract

Coxiella burnetii is the causative agent of the Q Fever zoonosis. It presents a worldwide occurrence and domestic ruminants are the most common hosts of *C. burnetii* and source of human infection. Dogs can also be infected, however, their role in the epidemiology of this bacterium is not clear.

This study was conducted on female dogs presented to veterinary medical centers from the North and Centre regions of Portugal between October 2020 and March 2021 to perform ovariohysterectomy. Surplus blood samples from rotine analysis and tissues obtained from routine cirurgical preocedures were used for serological and molecular testing. During the study period a total of 78 different animals were sampled (n=78).

Serums were tested for the presence of specific antibodies anti-*C. burnetii* using a commercial ELISA adapted for multi-species detection (ID Screen Q Fever Indirect Multispecies®, IDVet®). The results were expressed in S/P% values. An S/P% >50% were classified as positive. A total of 60 (n=60) animals were tested. The sample was composed of 30 pure-breed and 30 crossbreed dogs, with ages ranging from five months to fifteen years old. There was only one positive result (S/P%=54.96) corresponding to a six-year-old female dog living in a rural area. The observed exposure was 1.7% (95% CI: 0.3 to 9.1%). Another female dog with five years old living in a peri-rural area had a suspicious result (S/P=43%).

To detect *C. burnetii* DNA in the reproductive tissue, qPCR tests were performed in 67 canine uteri. A negative result was obtained in all the samples meaning that the presence of *C. burnetii* was not evidenced.

In the serological tests, the rate of exposure found in the tested dogs was low. It may suggest that the female dogs from the north and center regions of Portugal are not often exposed to the bacterium. On the other hand, the convenient sampling could not be representative of the population, compromising the conclusions by biased results.

However, the study and surveillance of *C. burnetii* infection in companion animals could be an asset to prevent outbreaks, and the transmission to owners and veterinarians contacting with infected animals.

Keywords: Q fever, pets, ELISA, qPCR, zoonosis

1. Introduction

Coxiella burnetii, the causative agent of zoonosis Q fever, is an obligate Gram-negative intracellular bacterium with a complex cycle (Angelakis & Raoult, 2010; Kazar, 2005).

The bacterium can survive in the environment for months due to its ability to survive in very adverse conditions (Kazar, 2005). The bacterium presents three different forms during it's cycle that includes large-cell variants (LCV), small-cell variants (SCV) and small dense cells (SDC). The SCV and SDC are considered as the resistant forms of *C. burnetii* in the environment allowing the bacteria to survive extracellulary as infectious particles. (Arricau-Bouvery & Rodolakis, 2005)

Q fever is a worldwide recognized disease that remains often underreported and its control is frequently neglected (Porter et al., 2011). So far, New Zealand is the unique country with no autochthonous cases reported, probably because of strict control measures and its geographic remoteness (Kazar, 2005). The largest outbreak of Q fever ever reported happened in the Netherlands from 2007 to 2011 where, more than 4000 human cases were reported (Schneeberger et al., 2014).

In humans, Q fever varies from an asymptomatic occurrence, mild symptomatic manifestation, or fatal (Angelakis & Raoult, 2010). The clinical manifestations of Q fever depend on the bacterial virulence potential but mainly on each immunological system and risk factors (Eldin et al., 2016). The symptoms include fever, pneumonia, hepatitis, cardiac pathology, skin rash, and neurological signs (Angelakis & Raoult, 2010).

In Portugal, the notification of Q fever is mandatory for humans (Santos et al., 2007) and despite the non mandatory notification to the National Veterinary Authoroty it is a notifiable disease to the World Organization for Animal Health (OIE, 2021).

In animals, the disease is often referred to as Coxiellosis. Normally it is asymptomatic, and animals act mostly as reservoirs of infection. In fact, in animals the infection can remain latent and later it can cause abortions (Kazar, 2005). This occurs due to the reactivation of the bacteria by the immunomodulation induced by the hormonal changes during pregnancy (Porter et al., 2011).

Domestic ruminants are the most common hosts of *C. burnetii* and the most important and common source of human infection (Kazar, 2005; Porter et al., 2011). Although it is known that other animal species can act in the transmission cycle of *C. burnetii* since this agent has already been found in mammals, insects, birds, reptiles, and even fishes (Kazar, 2005).

Ticks can also be infected and are considered an important vector and reservoir of *C. burnetii* in wildlife. They can spread bacteria to vertebrates through their bite in meals or even through the ingestion of their feces (Kazar, 2005).

The human infection occurs mainly through the inhalation of contaminated aerosols from the birth products, milk, urine, feces, saliva, or contaminated wool of the infected animals (Kazar, 2005; Angelakis & Raoult, 2010; Porter et al., 2011). The infection through the ingestion of raw milk or dairy products of infected animals is controversial and not considered a risk factor in *C. burnetii* outbreaks (Bogunović et al., 2018). People who have more contact whit animals like farmers, shepherds, veterinarians, slaughterhouse operators, and hunters are some of the most infected groups whit Q fever (Santos et al., 2007).

The contact with birth products is the most common way of transmission of the bacteria since *C. burnetii* presents a higher concentration in the uterus, placenta, vaginal fluids and secretions, and mammary glands (Laughlin et al., 1991; Angelakis & Raoult, 2010; Anastácio, 2019).

Human infection by farm animals is most common in rural areas. It is presumed that in urban areas*, C. burnetii* infection is more related to the transmission from infected pets like cats and dogs (Angelakis & Raoult, 2010).

Dogs can also be infected by the inhalation of contaminated aerosols, tick bites, and by the ingestion of birth products and raw milk from infected animals. The infection is often subclinical but later abortions or premature death of puppies has been associated with *C. burnetii* infection (Porter et al., 2011). In this context, companion animals can cause outbreaks of Q fever in humans, even if there is no history of contact with domestic ruminants (Cooper et al., 2011).

In Portugal, a lack of information regarding this disease is recognized mainly in animals bu also in humans which is probably due to an under-diagnosed and an underreport of cases (Santos, 2015).

The diagnosis of Coxiellosis is based on serology to detect the exposure to the agent. Immunofluorescent assay (IFA), Complement Fixation Test (CFT), and Enzyme-Linked Immunosorbent Assay (ELISA) are the most used diagnostic methods (Kazar, 2005; Tilburg et al., 2010).

PCR tests are used to detect the agent in biological samples. This direct test provides a specific, sensitive, and fast diagnosis of *C. burnetii* (Arricau-Bouvery & Rodolakis, 2005).

Given the role that companion animals may play in the transmission of *C. burnetii* to humans (Buhariwalla et al., 2013), this study aimed to evaluate the:

i) exposure to *Coxiella burnetii* in dogs from north and center of Portugal by ELISA testing.

ii) the presence of *C. burnetii* DNA in female uterine secretions/tissues by qPCR testing.

2. Materials and Methods

2.1. Study design

From October 2020 to March 2021, an observational descriptive study was conducted on dogs from the Center and North Regions of Portugal. A convenience sampling was conducted in female dogs submitted to ovariohysterectomy in specialized animal clinics which agreed to participate in the study from the region of Coimbra (n=2), Aveiro (n=2), and Porto (n=2) and under the written agreement of owners (Annex I). Thus, the surplus tissues obtained from surgeries and the surplus serum/plasma obtained during pre-anesthetic analysis or general blood panels before the surgery were also saved. The anonymous of the patients were kept, and the well-being rules of the animals were strictly followed and assured and none of the procedures was purposely done for this study.

The obtained samples were identified, and data were registered in a small questionnaire (Annex II) (*e.g.,* date of sample collection, area, age, breed, contact whit other animals, habitat area, and type of surgery).

2.2. Sampling procedures

Uterine samples (n=67) were collected from female dogs that were submitted to ovariohysterectomy surgery prophylactically or because they had some concomitant reproductive pathology. Small pieces of uterine tissue or swabs from uterine endometrium were kept in individual containers, at -20ºC and stored at the laboratory of Microbiology of Escola Universitária Vasco da Gama until analysis.

From the same individuals, blood was collected for routine analysis before surgery. Blood samples were collected to tubes whit EDTA or dry tubes and stored at 5ºC ± 3ºC until centrifugation. After centrifugation, the serum were frozen and stored at -20ºC until analysis.

2.3. Serological analysis

The detection of anti-*Coxiella burnetii* antibodies was conducted using a commercial ELISA kit, including phase I and phase II *C. burnetii* antigens obtained from infected ruminants and adapted for multi-species (ID Screen Q Fever Indirect Multispecies®, IDVet®). (Annex III and IV)

Results were obtained by reading the Optic Density (OD) at 450nm in the spectrometer. Validation was confirmed by i) a mean value of the Positive Control (PC) greater than 0,350; and ii) a ratio of the mean values of the PC OD to the Negative Control (NC) OD greater than 3. The positive and negative control were included in the kit.

The interpretation of the results was performed by analizing the sample to positive ratio (S/P%) wich was calculated using the following formula:

$$\text{S/P \%} = \frac{\text{OD sample} - \text{OD NC}}{\text{OD PC} - \text{OD NC}} \text{ x } 100$$

Results were categorised in four groups:

1) negative if S/P% ≤ 40%;
2) doubtful if 40% < S/P% ≤ 50%;
3) positive if 50% < S/P% ≤ 80%;
4) strong positive if S/P% > 50%.

2.4. Molecular analysis

The PCR testing for detection of *C. burnetii* was performed after DNA isolation from samples. Considering the frozen uterine pieces, about 25 mg of tissue was collected aseptically, using scalpel blades, and a group of 10 samples was pooled and homogenized in sterile Petri dishes. Twenty five miligrams (mg) of the previous homogenate was used for DNA extraction. Swabs of uterine endometrium were suspended in 1 mL of sterile phosphate buffered saline (PBS). In a 2 mL Eppendorf® tube, 200 µL of each swab was placed and vortexed. In the end, 200 µL was used for DNA extraction using QIamp DNA Mini Kit (Qiagen®] according to manufacturer instructions (Annex V).

A qPCR assay targeting *IS1111* was conducted on a CFX-96 thermocycler (Bio-Rad®) and using the commercial Taq-Vet™ *Coxiella burnetii* kit (Thermo Fisher Scientific LifeTechnologies®) to determine the cycle threshold (Ct) values following the manufacturer instructions (Annex VI). To confirm that no inhibitions of Taq Polymerase occurred samples were tested in duplicate. In one sample 5 µL of canine DNA was used and in the other sample, a mixture of 2.5 µL of canine DNA was associated with 2.5 µL of *C. burnetii* negative ruminant DNA. For each sample, the results were interpreted as shown in Table 1.

Table 1: *C. burnetii* PCR interpretation

Coxiella burnetii detector	Internal Positive *control* *C. burnetii* detector	Interpretation
Ct < 45	Ct < 45 or Ct > 45	Detected
Ct > 45	Ct < 45	Not Detected
Ct > 45	Ct > 45	Not Validated

2.5. Statistical analysis

For statistical purposes, simple logistic regression analysis was performed to explore associations between individual factors and response variables. Confidence limits for the proportions were estimated with 95% confidence intervals (CI) assuming a binominal exact distribution (EpiInfo version 3.5.4; Center for Disease Control and Prevention, Atlanta, GA, USA).

3. Results

3.1. Descriptive analysis

Of all the 78 sampled female dogs, only 60 (76.9%) were tested for ELISA. The mean age of tested animals was calculated in 52 months (SD = 46.6; ranging from five to 180 months). About 51.7% (n=31) of sampled animals were from Águeda and Coimbra; 50% (n=30) were obtained from crossbreed animals; 75% (n=45) were companion animals; 75% (n=45) from urban areas and 78.3% (n=47) lived with no other animal species. This analysis can be consulted in more detail in Table 2.

Also, 67 (85.9%) uterine samples were tested by qPCR. The mean age of animals was calculated at 52.4 months (SD = 48.5; ranging from five to 180 months). About 53.7% (n=36) of sampled animals were from Agueda and Coimbra; 53.7% (n=36) were obtained from crossbreed animals; 74.6%(n=50) were companion animals; 70.1% (n=47) from urban areas and 76.1% (n=51) lived with no other animal species. Table 4 systematizes this information.

3.2. Serological testing

Seropositivity of 1.7% (95% CI: 0.3 to 9.1%) was observed among the 60 tested female dogs as observed in Tables 2 and 3.

Table 2 – Descriptive characteristics of ELISA tested in female dogs.

Variables	Frequency n (%)	Seropositivity n (%)	* Confidence interval 95% (%)
Breed			
Crossbreeds	30 (50)	0	0
Labrador Retriever	7 (11.7)	0	0
French Bulldog	3 (5)	0	0
Beagle	2 (3.3)	0	0
Pinscher	2 (3.3)	0	0
German Shepherd	2 (3.3)	0	0
Yorkshire Terrier	2 (3.3)	0	0
Chihuahua	1 (1.7)	1 (1.7)	0.3 – 9.1
Dalmatian	1 (1.7)	0	0
Doberman	1 (1.7)	0	0
English Bulldog	1 (1.7)	0	0
Galgo	1 (1.7)	0	0
Golden Retriever	1 (1.7)	0	0
Great Dane	1 (1.7)	0	0
Jack Russel	1 (1.7)	0	0
Maltese	1 (1.7)	0	0
Cane Corso	1 (1.7)	0	0
Pug	1 (1.7)	0	0
Serra da Estrela	1 (1.7)	0	0
Area			
Águeda	16 (26.7)	1 (1.7)	0.3 – 9.1
Coimbra	15 (25)	0	0
Oliveira do Bairro	5 (8.3)	0	0
Figueira da foz	4 (6.7)	0	0
Valongo	4 (6.7)	0	0
Maia	3 (5)	0	0
Mortágua	2 (3.3)	0	0
Anadia	2 (3.3)	0	0
Arganil	2 (3.3)	0	0
Nisa	1 (1.7)	0	0
Sever do Vouga	1 (1.7)	0	0
Tondela	1(1.7)	0	0
Albergaria-a-Velha	1(1.7)	0	0
Aveiro	1(1.7)	0	0
Leiria	1(1.7)	0	0
Miranda do Corvo	1(1.7)	0	0
Purpose			
Companion	45 (75)	1 (1.7)	0.3 – 9.1
Guard	7 (11.7)	0	0
Work	3 (5)	0	0
Hunt	0	0	0
Kennel	5 (8.3)	0	0
Stray	0	0	0
Geographical area			
Rural	3 (5)	1 (1.7)	0.3 – 9.1
Peri-urban	12 (20)	0	0
Urban	45 (75)	0	0
Cohabitation			
Yes	13 (21.7)	0	0
No	47 (78.3)	1 (1.7)	0.3 – 9.1
Cause of surgery			
Elective OVH	42 (70)	0	0
OVH with Pyometra	7 (11.7)	1 (1.7)	0.3 – 9.1
OVH with Mastectomy	7 (11.7)	0	0
OVH with Hydrometra	1 (1.7)	0	0
OVH with Cesarian	2 (3.3)	0	0
OVH with Vaginal prolapse	1 (1.7)	0	0

*Calculated in http://vassarstats.net/

ELISA results are systematized in Table 2 and showed that exposure to *C. burnetii* occurred in one animal. An suspicion of exposure was observed in one other animal. The exposure occurred in a companion Chihuahua of 72 months old living with no cohabitants in a rural area in Águeda. The suspicion of exposure was observed in a companion mixed breed female dog whit 60 months old, living with no cohabitants, in a peri-rural area, in Tondela.

Table 3 – Descriptive statistics of *C. burnetii* antibodies in female dogs

Test category	Nr of animals	Mean age of animals (months)	Percentage	95% CI	Range of S/P%	Mean S/P value
Negative	58	50.4	0.966	0.88-0.99	0.13-17.22	8,11
Doubtful	1	60	0.017	0.03-0.091	43.78	43.78
Positive	1	72	0.017	0.03-0.091	54.96	54.96
Strong positive	0	n.a.	n.a.	n.a.	n.a.	n.a.

n.a. not apliable

3.3. Molecular testing

A total of 67 canine uteruses organized in seven pools of DNA samples were tested for the presence of *Coxiella burnetii* DNA. A negative result was obtained in all the samples (100%). The presence of *C. burnetii* was not evidenced in the studied female dogs. In Table 4 is possible to see the descriptive characteristics of the PCR tested dogs.

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Table 4 – Descriptive characteristics of PCR tested in female dogs.

Variables	Frequency n (%)	****C. burnetii*** DNA detection n (%)**
Breed		
Crossbreeds	36 (53,7)	0
Labrador Retriever	7 (10,4)	0
Beagle	3 (4,5)	0
Pinscher	3 (4,5)	0
French Bulldog	2 (3)	0
German Shepherd	2 (3)	0
Yorkshire Terrier	2 (3)	0
Chihuahua	2 (3)	0
Argentine Dogue	1 (1,5)	0
Boxer	1 (1,5)	0
Chinese Crested Dog	1 (1,5)	0
Dalmatian	1 (1,5)	0
Galgo	1 (1,5)	0
Golden Retriever	1 (1,5)	0
Great Dane	1 (1,5)	0
Jack Russel	1 (1,5)	0
Maltese	1 (1,5)	0
Serra da Estrela	1 (1,5)	0
Area		
Águeda	18 (26,9)	0
Coimbra	18 (26,9)	0
Anadia	10 (14,9)	0
Maia	4 (6)	0
Oliveira do Bairro	4 (6)	0
Valongo	4 (6)	0
Figueira da foz	3 (4,5)	0
Arganil	2 (3)	0
Albergaria-a-Velha	1 (1,5)	0
Aveiro	1 (1,5)	0
Mortágua	1 (1,5)	0
Miranda do Corvo	1 (1,5)	0
Purpose		
Companion	50 (74,6)	0
Guard	7 (10,4)	0
Work	3 (4,5)	0
Hunt	1 (1,5)	0
Kennel	6 (9)	0
Stray	0	0
Geographical area		
Rural	2 (3)	0
Peri-urban	18 (26,9)	0
Urban	47 (70,1)	0
Cohabitation		
Yes	16 (23,9)	0
No	51 (76,1)	0
Cause of surgery		
Elective OVH	47 (70,1)	0
OVH with Pyometra	7 (10,4)	0
OVH with Mastectomy	9 (13,4)	0
OVH with Hydrometra	1 (1,5)	0
OVH with Cesarian	1 (1,5)	0
OVH with Vaginal prolapse	1 (1,5)	0
OVH with Abortion	1 (1,5)	0

*Calculated in http://vassarstats.net/

4. Discussion

The laboratory diagnosis of *C. burnetti* infection should be done with a combination of serological and molecular tests (Wegdam-Blans et al., 2012). This method allows to confirm a positive diagnostic discarding false positives and negatives. To evaluate the exposure to *C. burnetii*, an ELISA test was used following the recommendations of European Food Safety Authorithy (EFSA) (2010) and OIE (2018). This test has the advantage of being easier to perform and presents a high sensitivity as it allows the analysis of multiple samples in the same assay, which permits "large scale studies" (Porter et al., 2011).

The ELISA antigen used for this experiment was collected from infected ruminants. It is described that the antigen source of the ELISA test may influence the results. The antigen obtained from European domestic ruminants isolates shows a higher sensitivity when compared to the Nine-mile antigen isolated from ticks (Arricau-Bouvery and Rodolakis, 2005).

qPCR tests were realized to detect *C. burnetii* DNA in the reproductive tissues of the tested animals. Since this bacterium has a higher concentration in the reproductive tissues, fluids and secretions (Angelakis & Raoult, 2010), a portion of the uterine endometrium was chosen as the sample for PCR testing. The qPCR has high sensitivity and specificity and it laso can be used in "large scale studies". It is very useful for early diagnosis because it can detect bacterial DNA before the development of specific antibodies (Porter et al., 2011; Wegdam-Blans et al., 2012). The positive and negative controls allowed to exclude the probability of DNA isolation error.

In this study, the seropositivity of the samples was very low, and the OD observed in the positive sample was also low. Despite the positive result in the serological test, no animal tested positive in the molecular test.. This can be explained by one of the following situations:

-The presence of IgG might result from a past infection which may lead to a positive result on ELISA test by detectionof IgG's antibodies in the sample, leading to a positive result in the ELISA test and a negative result in the PCR test (Wegdam-Blans et al., 2012).

- The type of sample chosen may not have been the most suitable since the bacteria presents high concentrations in birth products and most of the animals were not pregnant at the moment of sample collection (Van den Brom & Vellema, 2009).

-The concentration of DNA in the sample could be lower than the limit of detection by PCR.

Typically, *C. burnetii* infection is correlated with the proximity to domestic ruminants, since they are the principal source of human infection (Santos et al., 2007). Although dogs may present (mistakenly) a less important role in the transmission of Q fever, they can also be a reservoir and source of transmission to humans. Also, the probability of dogs being seropositive is ten times higher if they have closer contact with domestic ruminants (Boni et al., 1998). In one study mode with samples from 429 French military dogs from 5 different countries were detected 42 (9.8%) positive cases, and the dogs whith higher titles were in contact with sheep (Boni et al., 1998).

Outbreaks related to the transmission of *C. burnetii* from dogs to humans have already been described.The first one was described in 1989 in Canada. Six out of seven family members tested positive for *C. burnetii*. Their dog gave birth under the owner's bed and the puppies died. Later, *C. burnetii* DNA was isolated in the dogs' uterus (Laughlin et al., 1991). Another outbreak of *C. burnetii* was described in 1996 in Nova Scotia. Three family members had symptoms and were present during the birth of the puppies who quickly died after the birth. Two of the family members tested positive for *C. burnetii* and the dog's serology was also positive (Buhariwalla et al., 1996).In 2013, three veterinary nurses became ill and tested positive for *C. burnetii* after having direct contact whit a parturient female dog that also tested positive (Gibbons & White, 2013). All of this reports have in commun the fact that the infected people were near the parturient dog, also that the puppys died shortly after delivery or were already dead and that the dogs also tested positive to *C. burnetii.*

Dogs represent a role in the epidemiology of *C. burnetii* even though there is still a lack of information about this subject. The number of veterinarians working with companion animals and, once considered, low-risk populations diagnosed with Q fever is rising. This can be explained or co-related to the ever-growing close relationship between humans and dogs contributing to this interspecies transmissibility (Cooper et al., 2011).

In Portugal, some studies were conducted to analyze the seroprevalence of *C. burnetii* in dogs. A study developed at Centro de Estudos de Vetores e Doenças Infeciosas Doutor Francisco Cambournac proved that *C. burnetii* DNA was present in samples of several animals, ticks, milk, and wells. In the same study, the presence of *C. burnetii* DNA was confirmed in all samples of the studied groups, except in the samples of domestic animals (Santos, 2015).

In 1995, a study was carried out in a kennel in Setúbal where 104 dogs were tested for *C. burnetii,* revealing a prevalence of 4,8% of the population with antibodies anti-*C. burnetii* (Bacellar et al.). In an investigation project conducted in Lisbon, blood samples from 39 dogs were tested by IFA, and 3 cases (4,3%) were positive (Duarte, 2014). In central Portugal, a serological study was carried out on companion animals with samples collected between 2011 and 2014, revealing an exposure rate of 12,6% in 151 dogs tested (Anastácio, 2019).

Other studies aiming to estimate the prevalence of *C. burnetii* in dogs' populations were realized in other countries. Considering vector transmission by ticks, in Japan 261 ticks from 48 dogs from four different geographical areas were collected, and RT-PCR was performed to detect *C. burnetii* DNA in the ticks. All the results were negative (Andoh et al., 2013). Evaluating the risk of abortion and neonatal mortality, a PCR study made in Italy with samples from abortions and neonatal mortality from 94 female dogs, 100% were negative to *C. burnetii* (Stefanetti et al., 2018).

Similar to this study, serosurveys were realized in 1223 Australian dogs and a total of 43 (3.5%) were positive to *C. burnetii* (Shapiro et al., 2016). Also, in Montenegro, 259 dogs were tested for *C. burnetii* and 3 (1.16%) were positive (Laušević et al., 2019).

These studies show that the prevalence of *C. burnetii* in dogs is low as it was demonstrated in the present study. Neverless, Cooper et al (2011) reported an increase of the seroprevalence in Australian dogs from 16% (n=16) from 1984-1985 to 21.8% (n=21.6%) from 2006-2007 which may represent a public health threat and consequently might be related to the occurrence of outbreaks (Cooper et al., 2011). Moreover, the fact that this infection goes unnoticed in most cases as it presents an asymptomatic infection, or shows unspecified clinical signs, leads frequently to the underdiagnose and undervaluation of this infection (Santos, 2015).

5. Conclusion

Coxiella burnetii is considered a potential agent of bioterrorism and it is in group B of potential Bioterrorism agents/diseases of the Centers for Disease Control and Prevention (OIE, 2018). It has a wide range of hosts including dogs. These can be sources of infection for humans even when asymptomatic, aggravating the zoonotic potential of this bacteria. A few outbreaks related to canine transmission have already been reported. The diagnosis of *C. burnetii* in small animals has been neglectable for many years but nowadays it is generally accepted that *C. burnetii* infection should be considered in the differential diagnosis of reproductive disorders (Santos, 2015).

Q fever infection is a human and animal problem that is still often neglected (Santos, 2015). It should be noted that there is an obligation to notify human cases to the human health authorities and Portugal must inform the OIE regarding animal cases the animal notification of infection it is not mandatory for portugueses animal health authorities.

The lack of information about this disease represents a public health problem (Angelakis & Raoult, 2010) and consequently leads to non-implementation of preventive and control measures. It is important to be aware mainly if people have direct or indirect contact with animals, or if living near because transmission can occur easily since the bacterium shows high resistance in the environment(Georgiev et al., 2013).

It is known that Q fever is endemic in Portugal, especially in domestic ruminants (Santos, 2015). This study can prove that the seropositivity in dogs in the studied regions in Portugal is not high but the probability of occurrence of outbreaks by dogs should not be ignored. However, the lower prevalence of *C. burnetii* in this study and the convenience sampling do not allow comparisons with the general population in Portugal as well as the data recorded in the survey.

In a possible futher study, it may be possible to use the nine-mile tick antigen in the ELISA kit and compare the results obtained. Thus, it will be possible to assess whether the infection in female dogs is dependent on the domestic cycle or the wild cycle of the bacteria.

Considering the reproductive tissue as the tissue with the highest bacterial concentration, the presence of *C. burnetii* DNA in the testicular tissue of male dogs could also be evaluated along with a serological test.

Also, try to specify the habitat zone of the animals, better evaluating the proximity that the individuals had to ruminants.

Consider individuals, from whom the samples were collected, had ticks or if they had a history of reproductive pathology such as abortion or neonatal deaths.

Finally, taking into account the positive result of this study, perhaps try to assess whether there is any influence of the bacteria on the development of pyometras in female dogs.

There is still little information about this disease in dogs and its role in the transmissibility of the bacteria, clinical signs, and potential sources of infection, mainly in urban areas. More studies need to be made by increase sampling, the areas in study, and the spectrum of *C. burnetii* hosts (Andoh et al., 2013).

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Annexes

Annex I – Owner's agreement

Consentimento de recolha de amostra biológica para estudo académico

Eu, abaixo-assinado, declaro que tomei conhecimento do estudo académico em realização e que consinto a recolha de amostras biológicas do meu animal ________________________, submetido voluntariamente por mim a cirurgia eletiva.

Data_________________

Assinatura_______________

Annex II – Sampling questionnaire

ID:

<table>
<tr><th colspan="3">Identificação de amostra recolhida</th></tr>
<tr><td>Número de identificação:</td><td>Data/hora:</td><td>Espécie:
Can. ☐
Fel. ☐</td></tr>
<tr><td>Idade:</td><td>Género:</td><td>Raça:</td></tr>
<tr><td>Amostra complementar:
Soro ☐
Sangue total ☐</td><td>Cirurgia:</td><td>Motivo da Intervenção:</td></tr>
<tr><td>Habitat:</td><td colspan="2">Contacto com outras espécies:</td></tr>
<tr><td colspan="3">Dados complementares:</td></tr>
</table>

Annex III – ELISA Kit composition (ID Screen Q Fever Indirect Multispecies®, IDVet)

ELISA Kit composition	Others
Microplates coated whit phase I and II Coxiella burnetii antigens	Mono pipettes capable of delivering volumes of 5 µl, 100 µl, and 500 µl
Concentrated conjugate (10X)	Disposable tips
Positive control	96-well pre-dilution microplate
Negative control	Distilled water
Dilution Buffer 2	A manual or automatic wash system
Dilution Buffer 3	96-well microplate reader
Substrate Solution	Eppendorf's
Wash concentrate (20X)	EDTA and dry tubes
Stop Solution (0,5 M)	Sample bottles
	Blood samples and uteruses

Annex IV – ELISA protocol according to the ELISA kit (ID Screen Q Fever Indirect Multispecies, IDVet®) instructions

ELISA in serum or plasma samples (IDScreen® Q fever Indirect Multi-species):

1. Samples are tested at a final dilution of 1:50 as follows:
    a. In a 96 well pre-dilution microplate add:

- 5 µl of the negative control to wells A1 and B1

- 5 µl of the positive control to wells C1 and D1

- 5 µl of each sample in the remaining wells

- 245 µl of the Dilution Buffer 2 to each well

2. In the ELISA microplate, transfer:

- 100 µl of prediluted Negative Control to wells A1 and B1

- 100 µl of prediluted Positive Control to wells C1 and D1

- 100 µl of each prediluted sample in the remaining wells

3. Cover the plate and incubate 45 min ± 4 min at 21ºC (± 5ºC).

4. Empty the wells.

5. Wash each well three times with 300µl of wash solution. Avoid drying of the wells between washes.

6. Prepare the Conjugate 1X by diluting the concentrated conjugate 10X to 1:10 in Dilution Buffer 3

7. Add 100 µl of the Conjugate 1X to each well.

8. Cover the plate and incubate 30 min ± 3 min at 21ºC (± 5ºC).

10. Empty the wells.

11. Wash each well 3 times with 300 µl of wash solution. Avoid drying of the wells between washes.

12. Add 100 µl of the Substrate to each well.

13. Cover the plate and incubate 15 min ± 2 min a 21ºC (±5ºC) in the dark.

14. Add 100 µl of the Stop Solution to each well.

15. Read and record the OD at 450 nm.

Annex V - DNA Extraction using QiAmp DNA Mini-Kit®

1. In an Eppendorf® tube add:

a) 200 µL of the PBS homogenate of swabs or 25 mg of homogenate tissues

b) 180 µl of ATL buffer

c) 20 µl of Proteinase K

2. Vortex one minute.

3. Incubate at 70ºC for 30 minutes.

4. Vortex for some seconds.

5. Add 200 µl of AL buffer and vortex for 15 seconds.

6. Incubate at 70°C for 10 minutes.

7. Add 200 µl of ethanol at 100%.

8. Vortex for 15 seconds.

9. Identify the columns and transfer the Eppendorf® content to the columns.

10. Centrifuge for one minute at 15000 g.

11. Discard the collection tube and preserve the column by placing it in a new collection tube.

12. Add 500 µl of AW1 buffer.

13. Centrifuge for one minute at 15000 g.

14. Discard the collection tube and preserve the column by placing it in a new collection tube.

15. Add 500 µl of AW2 buffer.

16. Centrifuge for 1 minute at 15000 g.

17. Discard the collection tube and preserve the column by placing it in a new collection tube.

18. Centrifuge for three minutes at 15000 g.

19. Discard the collection tube and place the column in a 1,5 mL Eppendorf® tube.

20. Add 200 µl of AE buffer to elute the DNA. Allow to incubate for 1 minute at room temperature and centrifugate at 6000 g for 1 minute.

21. Store the sample at refrigeration temperature if used immediately or freeze at -20ºC or -80ºC if further analysis.

Annex VI – qPCR protocol according to TaqVetTM *Coxiella burnetii* – Relative Quantification®

The "Mix FQP" is ready to use

1. Vortex the tube with the «Mix FQP» and pipette 20 µl of «Mix FQP» for each sample to be tested in an Eppendorf® tube. Do not forget the positive and negative controls.

2. On the equipment, create a microplate plan as follows:

a) Use “ROX” as passive reference and create two detectors – FQ (*reporter* FAM, *quencher* TAMRA); IPC (*reporter* VIC, *quencher* TAMRA)

b) Assign the FQ detector and the IPC detector to each well

3. Dispense 20 µl of mix per sample to be tested on the microplate for real-time PCR. In the Negative Control well, place 25 µl of Mix.

4. Add to each microplate well the corresponding sample.

» External Positive Control: place 5 µl of Standard DNA (concentration 10^4 in qualitative tests and concentration 10^1 to 10^4 in quantitative tests)

» Samples: 5 µl of DNA extracted from the sample

5. Cover the plate and place it in the thermocycler whit the following program:

» Stage 1: 50°C – 2 minutes

» Stage 2: 95°C – 10 minutes

» Stage 3: 95°C – 15 seconds and then 60°C – 1 minute – 45 reps.

Annex VII – SARS-CoV-2 serological testing

As a complement to this study was also realized a SARS- CoV-2 serological testing in the same samples that were used for *C. burnetii* testing. The abstract, testing procedure and results are included below.

Annex VII a) - Abstract - Serosurvey in dogs and cats to screen for the exposure to SARS-CoV-2

Neves, S.; Anjos, S.; Madeira, B.; Neves, T.; Craveiro, H.; Esteves, P.; Vilhena, H.; Anastácio, S.

Purpose: Severe acute respiratory syndrome–coronavirus 2 (SARS-CoV-2) causes COVID-19, which was first reported in Wuhan, China. This RNA virus has highly efficient transmission. So far, sporadic cases of infection in pets have been described. Human to animal transmission seems to occur, but the role of pets in the transmission of the disease remains unclear. This study aimed to screen for the exposure to SARS-CoV-2 in dogs and cats, during the peak of SARS-CoV-2 human infection in Portugal occurred between October 2020 and March 2021.
Methods & Materials: A cross-sectional study was conducted in dogs and cats presented to veterinary medical centers from Portugal between October 2020 and March 2021, that required

blood sampling as part of their diagnostic plan. Only surplus serum or plasma samples were used in this research. Serum or plasma was tested for the presence of specific antibodies anti-SARS-CoV-2 using a commercial ELISA adapted for multi-species detection (ID Screen SARS-CoV-2 Double Antigen Multispecies®, IDVet). Laboratory results were expressed in S/P% values and samples with an S/P% <50% were classified as negative. A total of 107 animals were sampled (dogs n=60; cats n=47). The canine population was composed of 25 pure-breed and 35 crossbreed dogs, with ages ranging from 5 months to 15 years old. Cats were mainly of the domestic short-hair breed (n=45), with ages ranging between 6 months and 9 years old. The estimated rate of exposure was of 5.0% (95% CI: 1.71-13.7%) in dogs and 2.13% (95% CI: 1.18-14.26%) in cats. A doubtful result was obtained in 6.7% (95% CI: 2.6-15.9%) of dogs and in 4.26% (95% CI: 2.6-15.9%).

Results: To our best knowledge this is the first serosurvey conducted on pets in Portugal. Exposure to the agent has been evidenced in dogs and cats. Further studies must clarify the impact of exposure on animal health and the role of pets in spreading the virus.

Keywords: COVID-19, ELISA, pets,

Annex VII b) - ELISA protocol according to ID Screen SARS-CoV-2 Double Antigen Multispecies®, IDVet

Allow all reagents to come to room temperature (21ºC ± 5ºC) before use. Homogenize all reagents by inversion or vortexing.

1. Add:

    - 25 µl of the Dilution Buffer 13 to each well

    - 25 µl of the negative control to wells A1 and B1

    - 5 µl of the positive control to wells C1 and D1

    - 25 µl of each sample to be tested to the remaining wells

2. Cover the plate and incubate 45 min ± 5 min at 37ºC (± 2º C).

3. Empty the wells. Wash each well five times with at least 300µl of wash solution. Avoid drying of the wells between washes.

4. Prepare the Conjugate 1X by diluting the concentrated conjugate 10X to 1:10 in Dilution Buffer 13

5. Add 100 µl of the Conjugate 1X to each well.

6. Cover the plate and incubate 30 min ± 3 min a 21ºC (± 5ºC).

7. Empty the wells. Wash each well 5 times with 300 µl of wash solution. Avoid drying of the wells between washes.

8. Add 100 µl of the Substrate to each well.

9. Cover the plate and incubate 20 min ± 2 min at 21ºC (±5ºC) in the dark.

10. Add 100 µl of the Stop Solution to each well in the same order as in step 8, to stop the reaction.

15. Read and record the O.D. at 450 nm.

Annex VII c) - SARS-CoV-2 results

Results were obtained by reading the Optic Density (OD) at 450 nm in the spectrometer. Validation was confirmed by:

i) a mean value of the Positive Control (PC) greater than 0,350

ii) a ratio of the mean values of the PC OD to the Negative Control (NC) OD greater than 3.

The interpretation of the results was performed using the following formula:

$$\text{S/P \%} = \frac{\text{OD sample} - \text{OD NC}}{\text{OD PC} - \text{OD NC}} \text{ x } 100$$

Results were categorized into four groups:

5) negative if S/P% ≤ 50%;
6) doubtful if 50% < S/P% < 60%;
7) positive if S/P% ≥ 60%;

Of all the 78 sampled female dogs, only 60 (76.9%) were tested for ELISA. The mean age of tested animals was calculated in 52 months (SD = 46.3 years; ranging from five to 180 months). About 51.7% (n=31 of sampled animals were from Agueda and Coimbra; 50% (n=30) were obtained from crossbreed animals; 75% (n=45) were companion animals; 75% (n=45) from urban areas and 78.3% (n=47) lived with no other animal species. This analysis can be consulted in more detail in Table 5

Table 5 - Descriptive characteristics of SARS-CoV-2 ELISA tested in female dogs

Variables	**Frequency n (%)**	**Seropositivity n (%)**	*** Confidence interval 95% (%)**
Breed			
Crossbreeds	30 (50)	1 (1.7%)	0.3% - 8.86%
Labrador Retriever	7 (11.7)	2 (3.3%)	0.92% - 11.36%
French Bulldog	3 (5)	0	0
Beagle	2 (3.3)	0	0
Pinscher	2 (3.3)	0	0
German Shepherd	2 (3.3)	0	0
Yorkshire Terrier	2 (3.3)	0	0
Chihuahua	1 (1.7)	0	0
Dalmatian	1 (1.7)	0	0
Doberman	1 (1.7)	0	0
English Bulldog	1 (1.7)	0	0
Galgo	1 (1.7)	0	0
Golden Retriever	1 (1.7)	0	0
Great Dane	1 (1.7)	0	0
Jack Russel	1 (1.7)	0	0
Maltese	1 (1.7)	0	0
Cane Corso	1 (1.7)	0	0
Pug	1 (1.7)	0	0
Serra da Estrela	1 (1.7)	0	0
County			
Águeda	16 (26.7)	1 (1.7)	0.3% - 8.86%
Coimbra	15 (25)	2 (3.3)	0.92% - 11.36%
Oliveira do Bairro	5 (8.3)	0	0
Figueira da foz	4 (6.7)	0	0
Valongo	4 (6.7)	0	0
Maia	3 (5)	0	0
Mortágua	2 (3.3)	0	0
Anadia	2 (3.3)	0	0
Arganil	2 (3.3)	0	0
Nisa	1 (1.7)	0	0
Sever do Vouga	1 (1.7)	0	0
Tondela	1(1.7)	0	0
Albergaria-a-Velha	1(1.7)	0	0
Aveiro	1(1.7)	0	0
Leiria	1(1.7)	0	0
Miranda do Corvo	1(1.7)	0	0
Purpose			
Companion	45 (75)	2 (3.3)	0.92% - 11.36%
Guard	7 (11.7)	1 (1.7)	0.3% - 8.86%
Work	3 (5)	0	0
Hunt	0	0	0
Kennel	5 (8.3)	0	0
Stray	0	0	0
Geographical area			
Rural	3 (5)	0	0
Peri-urban	12 (20)	0	0
Urban	47 (75)	3 (5)	1.71% -13.7%
Cohabitation			
Yes	13 (21.7)	0	0
No	47 (78.3)	3 (5)	1.71-13.7%
Cause of surgery			
Elective OVH	42 (70)	1 (1.7)	0.3% - 8.86%
OVH with Pyometra	7 (11.7)	2 (3.3)	0.92% - 11.36%
OVH with Mastectomy	7 (11.7)	0	0
OVH with Hydrometra	1 (1.7)	0	0
OVH with Cesarian	2 (3.3)	0	0
OVH with Vaginal prolapse	1 (1.7)	0	0

*Calculated in http://vassarstats.net/

Table 6 – Descriptive statistics of *SARS-CoV-2* in female dogs

Test category	Nr of animals	Mean age of animals (months)	Percentage	*95% CI	Range of S/P	Mean S/P value
Negative	54	51.89	0.90	79.85% - 95.34%	0.09 – 0,49	0.32
Doubtful	4	24	0.067	2.62% – 15.93%	0.51 – 0.54	0.52
Positive	3	88	0.05	1.71% - 13.7%	0.73 - 0.97	0.86

*Calculated in http://vassarstats.net/

Annex VIII – Serosurvey of *Coxiella burnetii* in companion animals from Portugal

Abstract

Q fever is a zoonotic infection that regained worldwide interest from health authorities following the large-scale human outbreak of Q fever in The Netherlands from 2007 to 2011. Ruminants are considered the main reservoirs of human infection. *Coxiella burnetii* infection has also been reported in companion animals, however, their role in the epidemiology of this bacterium is still unclear. This study aimed to perform a serosurvey of *Coxiella burnetii,* the causative agent of Q fever, in companion animals from Portugal.

A cross-sectional study was conducted in dogs and cats presented to veterinary medical centres from the North and Centre regions of Portugal between October 2020 and March 2021, that required blood sampling as part of their diagnostic plan. Only surplus serum samples were used in this research. Sera were tested for the presence of specific antibodies anti-*C. burnetii* using a commercial ELISA adapted for multi-species detection (ID Screen Q Fever Indirect Multispecies®, IDVet). Laboratory results were expressed in S/P values (optic density of the sample / optic density of the positive control sample). Samples with an S/P value between 40% and 50% were considered suspicious, and samples with SP values >50% were classified as positive.

A total of 107 animals were sampled (dogs n=60; cats n=47). The canine population was composed by 25 pure-breed and 35 crossbreed dogs, with ages ranging from 5 months to 15 years old. Cats were mainly of the Domestic Short-Hair breed (n=45), with ages ranging between 6 months and 9 years old. The estimated exposure rate was of 1% (95% CI: 0.02-5.1%), meaning that only one positive result was obtained (1/107) with an S/P of 54.9%, corresponding to a six years old female dog living in a rural area. Another female dog with five years old living in a semi-rural area had a suspicious result (S/P=43%).

The rate of exposure found in pets was very low, and even inexistent in cats. This finding suggests that companion animals from the North and Center regions of Portugal are not often exposed

to the pathogen. However, the monitoring of *C. burnetii* infection in companion animals is a major tool to prevent human outbreaks, considering the zoonotic potential for owners and veterinarians contacting with infected animals, mainly dogs and cats from rural areas which often contact with livestock.