ESCOLA UNIVERSITÁRIA VASCO DA GAMA

MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

SARS-COV-2 AND COXIELLA BURNETII IN FEMALE CATS FROM THE CENTRAL REGION OF PORTUGAL

Suzi Marina Alves Neves

Coimbra, Julho de 2021

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“Equipped with his five senses, man explores the universe around him and calls the adventure Science.”

Edwin Hubble

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Submetido e aceite no ECVIM (European College of Veterinary Internal Medicine)

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

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Submetido e aceite no IMED (International Meeting on Emerging Diseases and Surveillance)

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

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LIST OF ACRONYMS AND ABBREVIATIONS

AB – Antibodies

ACE2 – Angiotensin Converting Enzyme II

AG – Antigens

AngII – Angiotensin II

CDC – Centers for Disease Control and Prevention

CECAV – Animal and Veterinary Research Centre

CIVG – Vasco da Gama Investigation Centre

CNC – Neurosciences and Cell Biology Centre

COVID-19 – Coronavirus disease 2019

CSG – *Coronaviridae* Study Group

Ct – Cycle Threshold

DNA – Deoxyribonucleic Acid

ECVIM – European College of Veterinary Internal Medicine

ELISA – Enzyme-Linked Immunosorbent Assay

EUVG – Vasco da Gama University School

HVBV – Baixo Vouga Veterinary Hospital

HVUC – University Veterinary Hospital of Coimbra

ICTV – International Committee on Taxonomy of Viruses

IMED – International Meeting on Emerging Diseases and Surveillance

LHAP – Histology and Anatomical Pathology Laboratory

OIE – Office International des Épizooties

OP – Optical Density

OVH – Ovariohysterectomy

PCR – Polymerase Chain Reaction

PBS – Phosphate Buffered Saline

RNA – Ribonucleic Acid

RT-PCR – Reverse Transcription Polymerase Chain Reaction

S/P% – Sample to Positive Ratio Percentage

SARS-CoV – Severe Acute Respiratory Syndrome Coronavirus

SARS-CoV-2 – Severe Acute Respiratory Syndrome Coronavirus 2

SARSr-CoV – Severe Acute Respiratory Syndrome-related Coronavirus

USA – United States of America

UTAD – University of Trás-os-Montes and Alto Douro

WAHIS – World Animal Health Information System

WHO – World Health Organization

TITLE PAGE

SARS-CoV-2 and *Coxiella burnetii* in female cats from the central region of Portugal

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RESUMO

SARS-CoV-2 é o agente patogénico responsável pela atual pandemia COVID-19 que começou em dezembro de 2019, em Wuhan, China. Até maio de 2021, cerca de 166 milhões de casos e mais de 3 milhões de mortes foram registados mundialmente. Este vírus de genoma RNA tem uma transmissão humano-a-humano altamente eficiente por meio de disseminação de gotículas respiratórias e aéreas. A manifestação clínica pode ser ausente ou leve, mas em alguns casos ocorre pneumonia severa. Os gatos geralmente não apresentam sinais clínicos atuando como hospedeiros silenciosos.

A febre Q é uma zoonose mundial causada pela bactéria *Coxiella burnetii*. A infeção em humanos é caracterizada por uma doença febril inespecífica que pode evoluir para quadros mais graves, como pneumonia e hepatite. Em ruminantes domésticos está associada a alterações reprodutivas, enquanto que em gatos é muitas vezes assintomática e, portanto, a maioria das infeções passa despercebida. A febre Q é endémica em Portugal, mas o papel dos gatos na epidemiologia da infeção não é claro.

Vários estudos demonstraram o potencial zoonótico dos gatos para ambos os agentes patogénicos. Este estudo teve como objetivo a pesquisa serológica de SARS-CoV-2 e a pesquisa serológica e molecular de *C. burnetii* em gatas da região centro litoral de Portugal.

Entre outubro de 2020 e março de 2021, um total de 47 gatas atendidas em centros de atendimento médico-veterinários no centro litoral de Portugal foram incluídas neste estudo. Amostras excedentes de soro ou plasma colhidas para realização de exames complementares, foram armazenadas e posteriormente testadas para a presença de anticorpos anti-SARS-CoV-2 e anti-*C. burnetii* com o método de ELISA. Em fêmeas sujeitas ao procedimento cirúrgico de ovariohisterectomia, foram realizados esfregaços uterinos para deteção molecular de *C. burnetii* através de PCR.

Em relação ao SARS-CoV-2, a taxa de seropositividade foi 2.1% (IC 95%: 0.4 a 11,1%) (n=1), correspondente a uma gata de seis meses que apresentou S/P%=62,1%. Foram ainda obtidos dois resultados duvidosos (4.3%; IC 95%: 1.2 a 14.3%), com valores de S/P%=52,7% e 50,5%. Relativamente à pesquisa de anticorpos e DNA de *C. burnetii,* não foram obtidos resultados positivos.

Na amostra estudada, a taxa de exposição em gatas foi considerada baixa para SARS-CoV-2 e inexistente para *C. burnetii*. No entanto, os gatos representam uma grande ameaça como agente zoonótico para humanos, evidenciando a importância da vigilância destes agentes, especialmente SARS-CoV-2 em gatos com proprietários positivos para COVID-19 e *C. burnetii* em gatos que contatem com ruminantes domésticos.

PALAVRAS-CHAVE

COVID-19, febre Q, ELISA, PCR, animais de companhia

ABSTRACT

SARS-CoV-2 is the pathogen behind the ongoing COVID-19 pandemic that began in December 2019 in Wuhan, China. By May 2021, close to 166 million cases and over 3 million deaths were recorded worldwide. This RNA genome virus has highly efficient human-to-human transmission through the dissemination of respiratory and aerial droplets. The clinical manifestation may be absent or mild, but in some cases severe pneumonia occurs. Cats usually do not show clinical signs, therefore may act as silent hosts.

Q fever is a worldwide zoonosis caused by the bacterium *Coxiella burnetii*. Infection in humans is characterized by a nonspecific febrile illness that can progress to more severe conditions, such as pneumonia and hepatitis. In domestic ruminants it is associated with reproductive abnormalities, whereas in most cats it displays no symptoms, therefore most infections go unnoticed. Q fever is endemic in Portugal but the role of cats in the epidemiology of the infection is unclear.

Several studies have demonstrated the zoonotic potential of cats for both pathogens. The aim of this study was the serological research of SARS-CoV-2 and the serological and molecular research of *C. burnetii* in female cats from the central coast region of Portugal.

Between October 2020 and March 2021, a total of 47 female cats attended in veterinary medical centres in the central coast region of Portugal were enrolled in this study. Surplus serum or plasma samples collected for clinical purposes were stored and subsequently tested for the presence of anti-SARS-CoV-2 and anti-*C. burnetii* antibodies using the ELISA method. In females submitted to ovariohysterectomy, uterine swabs were collected for molecular detection of *C. burnetii*.

Regarding SARS-CoV-2, the seropositivity rate was 2.1% (95% CI: 0.4 to 11.1%) (n=1), corresponding to a six-month-old cat who displayed a S/P% of 62.1%. Two doubtful results were also obtained (4.3%; 95% CI: 1.2 to 14.3%), with values of S/P%=52.7% and 50.5%. Regarding the investigation of antibodies or DNA of *C. burnetii*, no positive results were obtained.

In the studied sample, the exposure rate in female cats was considered low in SARS-CoV-2 and non-existent in *C. burnetii*. Nevertheless, cats still impose a big threat as a zoonotic agent for humans, thus the importance of surveillance of these pathogens. Especially SARS-CoV-2 in cats with COVID-19 positive owners and *C. burnetii* in cats that have contact with domestic ruminants.

KEYWORDS

COVID-19, Q fever, ELISA, PCR, companion animals

1. BACKGROUND

1.1. SARS-COV-2

Numerous incomprehensible pneumonia cases were reported in hospitals in Wuhan, China, during December 2019. Initially, all these patients had a connection to a seafood and live animal market in the city. Later, clustered cases arose in different locations other than the city market of Wuhan, as well as, in individuals with no record of traveling to Wuhan itself (Wu et al., 2020). In early January 2020, the etiological agent was isolated and identified as a coronavirus, subsequently named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by the International Committee on Taxonomy of Viruses (ICTV) study group due to its similarities on phylogeny, taxonomy and established practise to the severe acute respiratory syndrome coronavirus (SARS-CoV) that caused an outbreak of over 8000 cases in 2003 (Gorbalenya et al., 2020). During that same month, the World Health Organization (WHO) declared the outbreak a public health emergency of international concern and on the 11th March of 2020, this novel virus was declared a pandemic responsible for the coronavirus disease 2019 (COVID-19) (Boni et al., 2020).

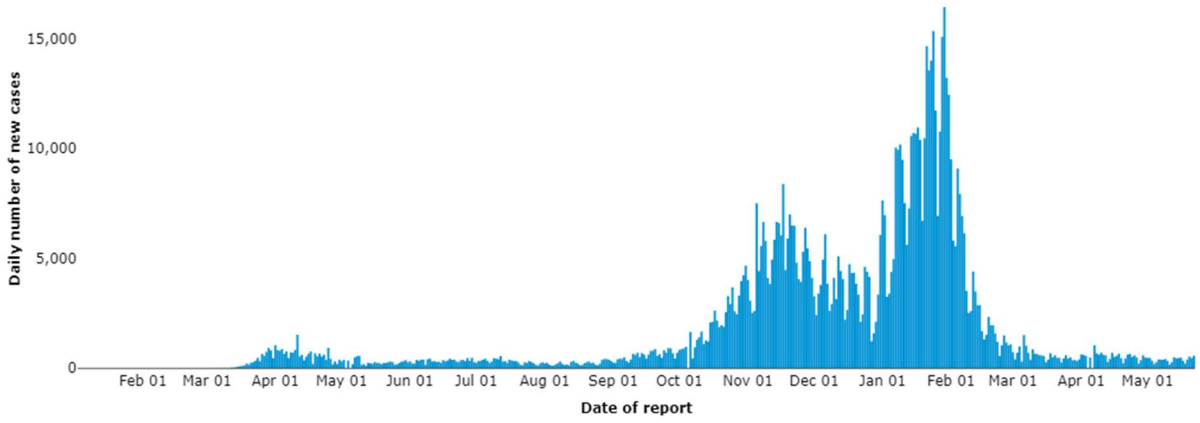
SARS-CoV-2 is a positive-sense single-stranded ribonucleic acid (RNA) virus responsible for the ongoing COVID-19 pandemic. It is included in the Coronaviruses category, *Sarbecovirus* subgenus and *Severe acute respiratory syndrome-related coronavirus* (SARSr-CoV) species, according to the classification of the *Coronaviridae* Study Group (CSG), a studying group of the ICTV (Gorbalenya et al., 2020). The SARSr-CoV species is characterized by having a high recombination rate and is mainly found in horseshoe bats (*Rhinolophus affinis*), which are believed to be the origin of SARS-CoV-2. Identically to the SARS-CoV, SARS-CoV-2 primary site of infection is the lungs and it also binds to the receptor angiotensin converting enzyme II (ACE2), utilizing it as a mechanism of cell entry (Zhou et al., 2020). ACE2 is responsible for regulating blood pressure and inflammation through conversion of angiotensin II (AngII) into other molecules that negate its effect. When SARS-CoV-2 binds itself to this receptor, levels of AngII rise causing damage to the blood vessel lining, inflammation and tissue injury (Esakandari et al., 2020).

This novel virus is highly pathogenic towards humans and has spread worldwide because of its highly efficient human-to-human dissemination. Respiratory droplet transmission is the primary route of diffusion, although it can also happen through airborne droplets and contact (Wu et al., 2020). Faecal-oral transmission is also a possibility, as the virus was detected in feces of infected people, however it is still uncertain that infection can occur after ingesting the virus. (Guo et al., 2021). Clinical characteristics of COVID-19 are non-specific, ranging from asymptomatic to severe life-threatening pneumonia. The most common symptoms include fever, dry cough and fatigue. Other less common symptoms are sore throat, headache, sputum production, myalgia, haemoptysis, diarrhoea, chills and loss of taste or smell (Hu et al., 2021).

Cats are extremely susceptible to SARS-CoV-2 infection, however they are typically asymptomatic so their infection can easily go unnoticed. (Gaudreault et al., 2020). It is believed that reverse-zoonosis is the most likely source of infection in domestic animals, on the other hand, the zoonotic potential of domestic animals has not been established yet. Nevertheless, infected cats shed the virus for several days and are capable of direct contact transmission to other cats (Bosco-Lauth et al., 2020).

The COVID-19 pandemic has persisted for over a year and a half now and is considered the greatest global health threat of the century (Hu et al., 2021). According to the WHO Coronavirus (COVID-19) Dashboard website, as of May 2021, it accumulated almost 166 million cases and over 3 million deaths globally. Meanwhile in Portugal, over eight hundred thousand cases were confirmed and more than seventeen thousand deaths registered (WHO, 2021b).

As mentioned before, multiples studies showed that this pathogen can infect cats which develop specific SARS-CoV-2 neutralizing antibodies (AB) (Bosco-Lauth et al., 2020; Fritz et al., 2021; Gaudreault et al., 2020; Patterson et al., 2020; Zhang et al., 2020). Given the great zoonotic potential of SARSr-CoV species, the close interaction among humans and pets makes cats a possible source of SARS-CoV-2 infection (Newman et al., 2020). However, SARS-CoV-2 epidemiology in cats remains largely unknown and limited studies have been carried out. To the best of our knowledge, no research has been carried out to date regarding this subject in Portugal. Hence, the importance of this study. Graphic 1 represents the daily number of new COVID-19 cases in Portugal from February 2020 to May 2021. This data was acquired from the WHO Coronavirus (COVID-19) Dashboard website. As shown on graphic 1, the peak of SARS-CoV-2 human infection in Portugal occurred between October 2020 and March 2021, which corresponds to the exact period of sampling collection for this study. Thus, a unique research opportunity is presented to explore the rate of seropositivity of SARS-CoV-2 in female cats from the central coast region of Portugal, which is the purpose of this study. The seropositivity will be determined using the enzyme-linked immunosorbent assay (ELISA) method.



Graphic 1: Daily number of new COVID-19 cases in Portugal from February 2020 to May 2021 (WHO, 2021a).

1.2. *COXIELLA BURNETII*

In the 1930s, an outbreak of a febrile illness emerged in Brisbane, Australia. Dr. Derrick, Director of the Laboratory of Microbiology and Pathology of Queensland Health Department, was assigned to investigate its cause. Despite his efforts, he was unable to make a final diagnosis, therefore it was named “Q fever” (Q for query). He then recruited more researchers, such as Sir Burnet, to help him isolate and identify the agent. Meanwhile, in Rocky Mountain Laboratory, Montana, Dr. Cox and Dr. Davis isolated the same organism from ticks and mistakenly associated it with *Rickettsia*. By the end of the decade, in 1938, Dr. Dyer, Director of the National Institutes of Health, examined the agent previously isolated by Cox and David and later became ill with that same agent. The pathogen was then isolated from his blood and compared to the agent present in spleens of infected mice sent by Burnet. It was a match and the Q fever agent was named *Coxiella burnetii* honouring Cox and Burnet findings (Marrie, 1995).

C. burnetii is a small intracellular gram-negative bacterium with a low dose infection and a high environmental resistance capable of aerosol dispersion, making it a potential biological weapon. It was previously classified as a member of the order Rickettsiales but later reclassified into the order Legionellales for its genetic and physiological differences (Greene, 2011). The life cycle consists of two forms, the obligate intracellular variant and the smaller sized extracellular variant which is not as metabolically active as the first one, but survives in the environment for two years and is resistant to heat and standard disinfectants (Plummer, 2015).

It is a worldwide zoonosis responsible for a self-limiting febrile illness associated with headache, but occasionally it may cause pneumonia and hepatitis, known as acute Q fever, which can evolve into more complicated chronic symptoms like endocarditis (Greene, 2011). Even though ticks like *Rhipicephalus sanguineus* are vectors of *C. burnetii*, most human infections occur through inhalation of the organism which makes pneumonia a common symptom (Ettinger et al., 2017). In spite of *C. burnetii* being known for generating infertility and sporadic abortion due to necrosis of the placenta in domestic ruminants (Plummer, 2015), most pregnant women remain asymptomatic although spontaneous abortion might occur in cases of untreated acute Q fever (Carcopino et al., 2009). The pathogen is excreted through urine, feces, milk and parturient discharges of infected animals like cattle, sheep, goats, and less frequently, cats and dogs. Thus it is recommendable the use of gloves and masks when handling such materials (Ettinger et al., 2017).

In cats the infection occurs commonly after contact with ticks, ingestion of contaminated carcasses or exposure to aerosols in contaminated environments. Most animals are asymptomatic therefore most infections go unnoticed but, symptoms like fever, anorexia and lethargy were shown in experimental infections and it may be linked to abortions in some cases (Nelson & Couto, 2015). These symptoms start two days after exposure to the pathogen and last around three days (Greene, 2011).

To date, the largest outbreak of Q fever was in the Netherlands with over 4000 cases reported from 2007 to 2010. It originated from abortion products at a dairy goat farm. During that period of time smaller outbreaks also appeared in Germany and Switzerland (Delsing et al., 2010).

The world distribution of Q fever reports in animals from 2005 to 2019 is represented in figure 1, which was obtained through the interactive interface of the Office International des Épizooties (OIE) and World Animal Health Information System (WAHIS) website.

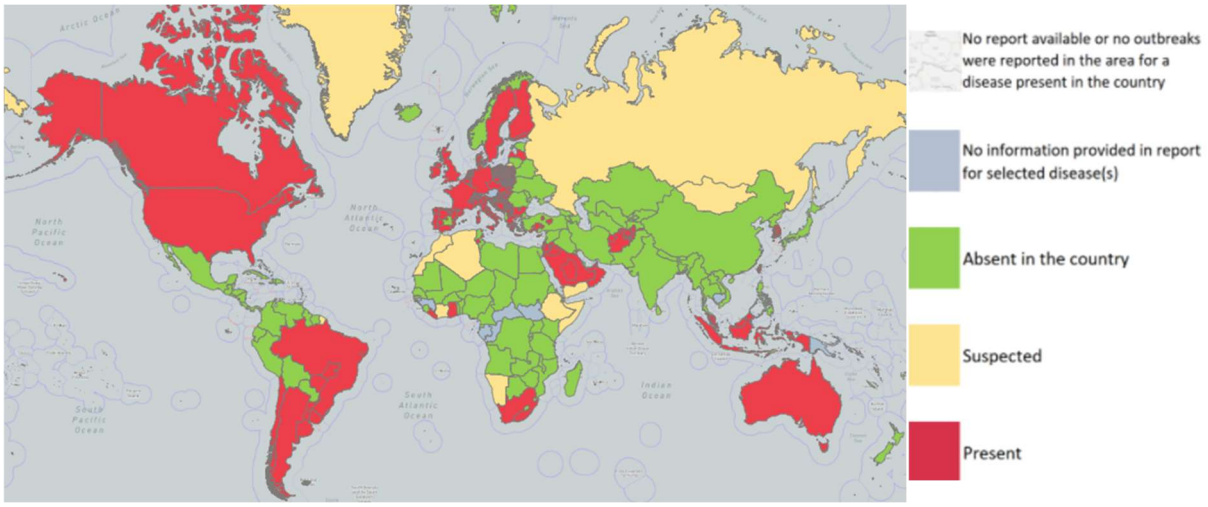


Figure 1: World distribution of Q fever reports in domestic and wildlife animals from 2005 to 2019 (OIE & WAHIS, 2021).

As shown in figure 1, Q fever is endemic in most of Europe and Portugal. The central region of the country presents an individual seroprevalence of 9.6% in small ruminants and 15.1% in cattle. The seroprevalence in both dogs and cats still requires more testing (Anastácio, 2019).

There have been several reports of Q fever outbreaks due to parturient cats (Kopecny et al., 2013; Kosatsky, 1984; Malo et al., 2018; Marrie et al., 1988), as well as, studies that show 5% to 13% seroprevalence in cats (Cairns et al., 2007; Cyr et al., 2021; Fujishiro et al., 2015; Khaled et al., 2021; Matthewman et al., 1997; Shapiro et al., 2015). Despite all this research, the possible zoonotic transmission from cats to humans still requires further study (Khaled et al., 2021).

The aim of this study is to determine the rate of seropositivity of *C. burnetii* in female cats from the central coast region of Portugal, as it is important to consider the danger that infected cats pose to humans. Seropositivity will be determined through the ELISA method. To evaluate the excretion properties of infected animals, uterine tissue will be tested for *C. burnetii* deoxyribonucleic acid (DNA) with the polymerase chain reaction (PCR) technique.

2. MATERIALS AND METHODS

2.1. STUDY DESIGN AND SAMPLING APPROACH

A cross-sectional study was conducted in the central coast region of Portugal over a period of six months. For sample size calculation, the domestic cat population was estimated according to Alves et al. (2005). Additionally, in agreement with Thrusfield (2007), an expected prevalence of 17.2% (Anastácio, 2019) and an absolute precision of 10% with a confidence interval of 95% were considered. The calculation using the software WinEpiscope (WinEpi: Working IN EPIdemiology) resulted in a minimum sample size of 55 animals. Convenience sampling was performed in female cats attending two veterinary hospitals and two veterinary clinics located in the central coast region of Portugal.

Samples were collected between October 2020 and March 2021, making a total of six months. Only female cats older than five months were included in this study. Furthermore, only animals subjected to blood collection for routine procedures and submitted to ovariohysterectomy (OVH) were enrolled in this research, always under the consent of the animals' owners (annex III). The surplus blood was centrifuged and the serum and plasma were stored individually in Eppendorf® tubes at -20ºC. Only for the purpose of the *C. burnetii* study, uterine swabs and a sample of uterine tissue were collected and inserted into sterile screw capped tubes and preserved at a temperature of -20ºC. The rest of the uterine tissue was placed under formalin in a sterile cup at room temperature. All the samples were properly identified and kept in the same designated conditions until further processing. Moreover, information regarding age, breed, habitat, cohabitants, contact with other animal species and the reason for the surgical intervention were obtained from medical records or the cats' owners.

2.2. SEROLOGICAL TESTING

2.2.1. ANTI-SARS-COV-2 ANTIBODIES

Serum or plasma samples were tested in duplicate using the commercial indirect ELISA kit, ID Screen® SARS-CoV-2 Double Antigen Multi-species ELISA (ID-Vet, France), following the manufacturer's instructions (annex IV). This is a double antigen ELISA test that detects AB directed against the nucleocapsid of SARS-CoV-2 in animal serum, plasma or whole blood. The sample to positive ratio percentage (S/P%) was achieved following the recommendations of the fabricator using the optical density (OD) values obtained with the following formula: $S/P\% = ((OD_{samples} - OD_{negatives}) \div (OD_{positives} - OD_{negatives})) \times 100$. According to the producer interpretation values, samples were considered negative if S/P% ≤ 50%, doubtful if S/P% > 50% and ≤ 60% and positive if S/P% > 60%. These procedures were performed at the laboratory of Vasco da Gama University School (EUVG).

2.2.2. ANTI-*C. BURNETII* ANTIBODIES

The serum or plasma samples were tested in duplicate for anti-*C. burnetii* AB with a commercial indirect ELISA kit, ID Screen® Q Fever Indirect Multi-species (ID-Vet, France), according to the producer's instructions (annex V). The wells in this kit are impregnated with phase I and II of *C. burnetii* antigens (AG) that form antigen-antibody complexes if anti-*C. burnetii* AB are present. The S/P% was determined through the OD values of the wells and implementing the formula given by the manufacturer: $S/P\% = ((OD_{samples} - OD_{negatives}) \div (OD_{positives} - OD_{negatives})) \times 100$. Following the fabricator directions, samples with S/P% ≤ 40% were considered negative, doubtful when S/P% > 40% and ≤ 50%, positive when S/P% > 50% and ≤ 80% and strongly positive when S/P% > 80%. These tests were executed at the laboratory of Vasco da Gama University School (EUVG).

2.3. MOLECULAR ANALYSIS

The PCR test for detection of *C. burnetii* was performed after DNA isolation from the samples. Considering the frozen uterine pieces, about 25mg of tissue were collected aseptically, using scalpel blades, and a group of ten samples was pooled and homogenized in a sterile Petri dish. Subsequently, 25mg of the previous homogenate were used for DNA extraction. Swabs of uterine endometrium were suspended in 1ml of sterile phosphate buffered saline (PBS), then 200µL of each swab were placed in a 2ml Eppendorf® tube and vortexed. Finally, 200µL were used for DNA extraction using the QIAamp DNA Mini Kit, Qiagen® (Isaza, Portugal), according to manufacturer instructions (annex VI). A real-time PCR assay targeting an insertion sequence used for detecting *C. burnetii*, the IS1111, was conducted on a CFX-96 thermocycler (Bio-Rad®). The commercial Taq-Vet™ *Coxiella burnetii* kit Lifetechnologies® (United States of America (USA)) was used to determine the cycle threshold (Ct) values following the producer directions (annex VII). These procedures were performed at the Faculty of Pharmacy at the University of Coimbra.

2.4. STATISTICAL ANALYSIS

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

3. RESULTS

3.1. POPULATION CHARACTERISTICS

For statistical analysis purposes, information was gathered about each individual cat including age, breed, habitat, cohabitants, contact with other animal species and the reason for the intervention (OVH) from medical records or the cats' owners (table 1).

A total of 47 female cats were enrolled in this study with ages ranging from five months to nine years old. However only 13 cats (27.7%) were older than one year, which means that 72.3% (n=34) of the cats were not considered adults yet. Cats were mostly of the Domestic Shorthair breed (n=45, 95.7%), with only one Persian cat and one Scottish Straight cat. Their habitat was mainly urban areas (n=30, 63.8%), while some were semi-urban (n=17, 36.2%). Rural cats were not included in this study. 38.3% of the cats (n=18) were known to have cohabitants and 17% (n=8) were known for not having any cohabitants, whereas the rest of the cats had no information regarding the subject (n=21, 44.7%). In regard to having contact with other animal species, 25.5% (n=12) were believed to be true and 17% (n=8) were believed to be false, while 57.5% (n=27) were not sure, however none of the cats enlisted in this study were known to have contact with domestic ruminants. The main reason behind the surgical procedure of OVH was for pregnancy prevention (n= 44, 93.6%).

Table 1: Frequency table representing the variables of age, breed, habitat, cohabitants, contact with other animal species and the reason for the intervention (OVH) of the 47 cats enrolled in this study.

VARIABLES	CATEGORIES	NUMBER OF CATS
Age	5-11 months	34
	1-5 years	10
	> 5 years	3
Breed	Domestic Shorthair	45
	Persian	1
	Scottish Straight	1
Habitat	Urban area	30
	Semi-urban area	17
	Rural area	0
Cohabitants	Yes	18
	No	8
	Unknown	21
Contact with other animal species	Yes	12
	No	8
	Unknown	27
Reason for the intervention (OVH)	Pregnancy prevention	44
	Mastectomy	2
	Pyometra	1

3.2. ELISA RESULTS

3.2.1. SARS-COV-2

SARS-CoV-2 neutralizing AB were detected in three of the 47 tested cats (6.4%; 95% CI: 2.2-17.2%), however two of them were in the doubtful result range, thus only one seropositive result was obtained (2.1%; 95% CI: 0.4-11.1). The positive result belongs to a six-month-old domestic short-haired cat from a semi-urban area, which was sampled on the 9th of December 2020 and displayed a S/P% value of 62.1%. The highest doubtful result has a S/P% value of 52.7% and was obtained from a six-year-old domestic short-haired cat from an urban area sampled on January 5, 2021. The lowest doubtful result has a S/P% value of 50.5% and comes from a domestic short-haired cat less than a year old from a semi-urban area. The exact date of collection of this sample is unknown. The two younger cats underwent surgery (OVH) for pregnancy prevention reasons. The six-year-old cat underwent a mastectomy, in addition to OVH, for presenting multiple mammary nodules.

3.2.2. *COXIELLA BURNETII*

Serum and plasma tested with ELISA for *C. burnetii* AB exhibited no significant differences in the OD of the duplicates, meaning that it was not possible to identify any positive result with a S/P% value higher than 40% (doubtful result) or 50% (positive result).

3.3. PCR RESULTS

A total of 47 feline uteri organized into five pools of DNA samples were tested for the presence of *C. burnetii* DNA. A negative result was obtained in all samples, which means that the existence of *C. burnetii* was not evidenced in the studied female cats.

4. DISCUSSION AND CONCLUSIONS

4.1. SARS-COV-2

Identifying every possible transmission route of an emerging pathogen is tremendously important to develop control measures that prevent its spread (Boni et al., 2020). The SARSr-CoV species have a huge zoonotic potential, yet the information regarding their seroprevalence in animals is still scarce. In particular, domestic animals may pose a major threat due to their close contact with humans (Bosco-Lauth et al., 2020). A study was conducted to determine the susceptibility of domesticated and laboratory animals to SARS-CoV-2 infection. Pigs, chickens and ducks showed no susceptibility to the virus, while dogs displayed low susceptibility. Contrarily, cats and ferrets demonstrated a high susceptibility. Additionally, massive lesions in the nasal and tracheal mucosa epitheliums and lungs were found on the post-mortem histopathologic exam of the cats, indicating that the virus can efficiently replicate within that host. In that same study, it was also found that younger cats are more vulnerable to infection and that the virus can be transmitted between cats through airborne droplets. Considering that cats are able to shed the virus, surveillance for SARS-CoV-2 in cats should be considered as a means to monitor COVID-19 (Shi et al., 2020).

The first reported case of SARS-CoV-2 in companion animals in the USA took place in April 2020, where two domestic cats from different households developed symptoms and subsequently tested positive in the reverse transcription polymerase chain reaction (RT-PCR) test performed in nasal and oropharyngeal swabs collected at the veterinary clinic. Both cats cohabited with a COVID-19 infected person, each separately in their respective household, suggesting a reverse-zoonotic transmission. Symptoms resembled those of a respiratory illness, namely, sneezing, coughing, nasal and ocular discharge and lethargy (Newman et al., 2020). On the other hand, experimental infection of domestic cats has shown that, generally, cats are asymptomatic, although extremely susceptible to infection and capable of shedding the virus for several days, in addition to developing AB that prevent reinfection. This fact makes SARS-CoV-2 infection in cats easily overlooked, therefore there is a possibility that cats may function as silent intermediate hosts (Bosco-Lauth et al., 2020; Gaudreault et al., 2020). So far, there is no clear evidence that animals play an important role in SARS-CoV-2 transmission to humans. However, the USA Centers for Disease Control and Prevention (CDC) recommends that people with suspected or confirmed COVID-19 avoid contact with animals during their illness period as a precautionary means (Newman et al., 2020).

Since the emergence of this novel virus, several studies have been carried out to determine the seroprevalence of SARS-CoV-2 in animals throughout the world. In France, a higher prevalence of SARS-CoV-2 AB has been found in cats and dogs living with people who have tested positive for COVID-19 when compared to pets of owners with an unknown COVID-19 status. This finding suggests that reverse-zoonosis is the most likely cause of infection in companion animals. Moreover,

cats were also found to have a higher seroprevalence than dogs, which is in line with previous research (Fritz et al., 2021). In Italy, an identical study was performed obtaining similar results, however, only dogs were found to have higher SARS-CoV-2 AB seroprevalence in COVID-19 positive households. This result may be explained by the fact that people have a closer interaction with dogs than with cats (Patterson et al., 2020). During the outbreak in Wuhan, serological research confirmed the presence of SARS-CoV-2 AB in serum samples of cats using the ELISA method. All samples were collected after the outbreak, also suggesting that the virus was transmitted from humans to cats (Zhang et al., 2020). Contrarily, no sign of SARS-CoV-2 infection was found in a cross-sectional study conducted in cats and dogs that lived in close contact with two veterinary students who tested COVID-19 positive. This suggests that the rate of transmission from humans to cats is probably low. Given this contradictory result, more research in this subject is recommended (Temmam et al., 2020).

In this study, one positive result (S/P%=62.1%) and two doubtful results (S/P%=52.7% and 50.5%) were found in a pool of 47 cats, achieving a SARS-CoV-2 rate of exposure of 6.4% if the doubtful results are considered and 2.1% if only positive results are considered. The age of these three cats ranged from six months to six years and all of them were of the Domestic Shorthair breed. Since only two cats out of the 47 total were of a different breed, conclusions regarding the susceptibility of the breed cannot be taken. No symptoms were mentioned by the pet owners at the time of the pre-surgical appointment, excluding the mastectomized cat which presented symptoms corresponding to mammary nodules complications, such as, anorexia, lethargy and weight loss, suggesting that those clinical signs were not related to the SARS-CoV-2 infection. Information concerning the COVID-19 status of the pet owners is not known in this study, for that reason it is not possible to conclude on the possibility of reverse-zoonosis transmission. As no other research of SARS-CoV-2 was found in Portugal, it is not possible to compare the results within the country. Nevertheless, this study demonstrated that cats are indeed susceptible to SARS-CoV-2 infection, therefore further testing is recommended to better assess the seroprevalence of SARS-CoV-2 in cats across the entire country and better understand the role they play in the spreading of this novel virus.

In conclusion, in spite of the sampling period coinciding with the peak of CODIVD-19 cases in Portugal, the exposure rate found in urban or semi-urban female cats from the central coast region of Portugal to SARS-CoV-2 was very low in this study. This can be justified by the fact that the sampling area did not have the highest incidence rate of COVID-19 in the country. It can also be explained due to people being more cautious with the use of masks and hygiene care, or cats being very independent animals that do not often enjoy close contact with humans for a long period of time. Nevertheless, this study has shown that cats are able to develop SARS-CoV-2 AB without displaying clinical signs noticeable by the pet tutors. This finding suggests that the infection can be easily unnoticed and, due to the zoonotic potential involved, monitoring of SARS-CoV-2 in domestic animals is extremely important in order to prevent new sources of infection. Especially in cats as they were shown to be highly susceptible in multiple studies. It is also advisable for COVID-19 positive people to avoid contact with their pets during the period of illness, since it is believed that reverse-zoonosis is

the main route of transmission to cats and other animal companions. Despite all the studies done to date, there is still a massive lack of knowledge worldwide in regard to this new virus, for that reason, it is of utmost importance that similar tests and other SARS-CoV-2 related tests are carried out in all domestic animals, cats included, in order to better comprehend this pathogen and discover new and more efficient ways of fighting it. Studies regarding the zoonotic transmission from domestic animals to humans seem to be specially lacking.

4.2. *COXIELLA BURNETII*

C. burnetii is currently ranked as a Category B bioterrorism agent by the USA CDC due to its highly contagious and resistant profile (Seshadri et al., 2003). Primarily it is considered an occupational hazard for veterinarians, farmers, workers in contact with dairy products or domestic ruminants and laboratory personnel who manipulate *C. burnetii* (Skerget et al., 2003). Nevertheless, it has been detected in the blood, urine and genital tract of infected cats and parturient cats have been identified as the source of infection in past outbreaks of human Q fever (Kopecny et al., 2013). The first association happened in Nova Scotia, Canada, in 1982, where all members of a house and its visitors fell ill and developed Q fever after the house cat gave birth to kittens, subsequently the cat tested positive for *C. burnetii* (Kosatsky, 1984). In the same province, in 1985, 25 cases of Q fever were identified after being in contact with a queen that gave birth to stillborn kittens (Marrie et al., 1988). During 2010 in Sydney, Australia, nine cases were reported in a small animal veterinary clinic after a caesarean section was performed on a seropositive breeding queen (Kopecny et al., 2013). From October to December of 2016 at an animal refuge and veterinary clinic in southeast Queensland, Australia, seven cases were diagnosed after the euthanasia of a cat and its litter (Malo et al., 2018). Taking all these cases into consideration, it is recommended that parturient queens are handled with the utmost hygienic care to prevent new Q fever occurrences (Ettinger et al., 2017). This also emphasises the importance of testing cats to this pathogen, even when they are asymptomatic (Skerget et al., 2003).

Multiple studies have been carried out with the objective of investigating the exposure of cats to *C. burnetii*. In the late 1990s, an investigation conducted with the indirect fluorescence method, tested 12.6% positives in a pool of 119 cats in Zimbabwe, southern Africa, and 2% out of 52 cats in south Africa (Matthewman et al., 1997). Between June 2002 and October 2003 in north-central Colorado, USA, *C. burnetii* DNA was found in 8.5% of the uterine samples of healthy cats out of 47 total (Cairns et al., 2007). An article published in 2015 showed a seroprevalence of 5.2% in a sample of 712 cats in eastern Australia (Shapiro et al., 2015). A study directed in the USA achieved a prevalence rate of 8.1% PCR positive results in a pool of 37, which was similar to the estimated number of 8.5% prevalence on client-owned cats in the area (Fujishiro et al., 2015). In Cairo, Egypt, a molecular study published this year, found a seroprevalence of 7.5% in a total of 40 cats. Furthermore all the positive results came from birth fluids of parturient queens (Khaled et al., 2021). In Portugal, a serological

study recently carried out in the central region of the country, showed an exposure of 17.2% of the 29 cats studied, all of them living in rural areas. However the presence of DNA was not evidenced (Anastácio, 2019). Despite these researches demonstrating the presence of *C. burnetii* in cats, in this study it was not possible to assess the exposure of cats to the agent, as no positive results were found.

The lack of positive results in this study may be justified by the fact that the majority of cats that participated in this study were from urban areas and had no known connection with domestic ruminants, which are the primary reservoirs for infection (Greene, 2011). In Japan, a study was performed to compare the difference in seroprevalence of *C. burnetii* in stray cats versus pet cats. Out of 36 stray cats, 15 had positive results, achieving a seroprevalence of 41.7%. In comparison, in a group of 310 pet cats, only 44 tested positive, reaching a seroprevalence of 14.2%. These findings indicate that the prevalence of this pathogen differs according to the living environments of the cats (Komiya et al., 2003). In the United Kingdom, a study was conducted that included only rural or semi-rural domestic outdoor cats with hunting habits. The seroprevalence was 61.5% in a sample of 26 cats. This result suggests that, in addition to the living environment, lifestyle habits may also impact the prevalence of this pathogen (Meredith et al., 2015). Including a larger and more diverse sample of cats will give a better idea of the true seroprevalence of *C. burnetii* in cats from the central coast region of Portugal, thus the importance of performing more similar studies.

In conclusion, the absence of positive results in this study suggests an inexistent exposure of female urban or semi-urban cats to *C. burnetii* in the central coast region of Portugal. However, the total of cats enrolled in the study was limited to 47 and restricted in terms of diversity. Therefore, more testing is necessary to allow better conclusions regarding the subject of seroprevalence of *C. burnetii* in cats from the central coast region of Portugal. Furthermore, the data acquired suggests that female cats from the central coast region of Portugal that live in urban or semi-urban areas and have no contact with domestic ruminants are not a big threat when it comes to the zoonotic potential for people, as they are not often exposed to the pathogen. Regardless, the monitoring of *C. burnetii* in companion animals, including cats, is still important in order to prevent outbreaks of Q fever in the future. Especially companion animals who had contact with domestic ruminants or livestock living spaces even if for a short period of time, for example, going on holidays to a rural area. Moreover, the use of gloves and masks is recommended when manipulating parturient discharges of cats, as it has been proven in the past to be a source of infection for people.

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ANNEXES

Annex I – 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.

Annex II – Serosurvey of SARS-CoV-2 in dogs and cats from Portugal

Abstract

Purpose: Severe acute respiratory syndrome–coronavirus 2 (SARS-CoV-2) causes COVID-19, which was first reported in humans in 2019, in Wuhan, China. This RNA virus has highly efficient transmission. Sporadic cases of infection in pets have been described. Human to animal transmission seems to occur, however, the epidemiologic role of pets remains unclear. This study aimed to screen dogs and cats from the North and Centre regions of Portugal for the exposure to SARS-CoV-2, during the peak of SARS-CoV-2 human infection in Portugal, which occurred between October 2020 and March 2021.

Methods & Materials: A cross-sectional study was conducted in dogs and cats presented to veterinary medical centres from Portugal between October 2020 and March 2021, that required blood sampling as part of their diagnostic plan. Only surplus sera or plasma samples were used in this research. Sera or plasma were tested for the presence of specific antibodies anti-SARS-CoV-2 using a commercial ELISA adapted for multi-species detection (ID Screen SARS-CoV-2Double Antigen Multispecies®, IDVet). Laboratory results were expressed in S/P values and samples with an S/P% ≥ 60% were classified as positive.

Results: A total of 107 animals were sampled (dogs n=60; cats n=47). The canine population was composed by 25 purebred and 35 crossbreed dogs, with ages ranging from 5 months to 15 years of age. 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 (n=3) and 2.13% (95% CI: 1.18-14.26%) in cats (n=1). A doubtful result (50≥S/P%<60%) was obtained in 6.7% (95% CI: 2.6-15.9%) of dogs (n=4) and in 4.26% (95% CI: 2.6-15.9%) of cats (n=3).

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

Annex III – Animal owner permission and questionnaire

(A preencher pela entidade competente) Número de amostra: _____ Data de colheita: __/__/____ Número do animal: _____

O presente inquérito tem como objetivo um estudo referente à prevalência de SARS-COV-2 e *Coxiella burnetii* em animais de companhia. SARS-CoV-2 é o agente patogénico responsável pela atual pandemia COVID-19. *Coxiella burnetii* é um agente zoonótico que causa febre Q em humanos. Os dados recolhidos serão usados para fins científicos e não serão partilhados com terceiros. A identidade dos participantes não será revelada. Agradecemos a sua cooperação no preenchimento dos dados referentes ao seu animal.

Nome do animal: _____

Espécie: Cão __ Gato __ Raça: _____ Idade: _____

Tipo de ambiente de habitação: Área urbana: ___ Área semiurbana: ___ Área rural: ___

Coabitantes: Sim: __ Se sim, Quais? _____ Não: ___

Contato com outras espécies animais: Sim: __ Se sim, Quais? _____ Não: __

Motivo da intervenção cirúrgica (ovariohisterectomia): _____

Eu, _____, declaro que dou o meu consentimento para a recolha e uso de sangue e útero do meu animal de companhia para fins científicos.

Data: __/__/____

Assinatura: _____

ANNEX IV – Indirect ELISA kit for serum or plasma (ID Screen® SARS-CoV-2 AG Multi-species)

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

1. Add:
    a. 25µl of Dilution Buffer 13 to each well;
    b. 25µl of the Negative Control to wells A1 and B1;
    c. 25µl of the Positive Control to wells C1 and D1;
    d. 25µl of each sample to be tested to the remaining wells.
2. Cover the plate and incubate 45min (± 5min) at 37ºC (± 2ºC).
3. Empty the wells. Wash each well 5 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 100µl of the Conjugate 1X to each well.
6. Cover the plate and incubate 30min (± 3min) at 21ºC (± 5ºC).
7. Empty the wells. Wash each well 5 times with at least 300µl of Wash Solution. Avoid drying of the wells between washes.
8. Add 100µl of the Substrate Solution to each well.
9. Cover the plate and incubate 20min (± 2min) at 21ºC (± 5ºC) in the dark.
10. Add 100 µl of the Stop Solution to each well, in the same order as in step No. 8, to stop the reaction.
11. Read and record the OD at 450nm.

Annex V – Indirect ELISA kit for serum or plasma (ID Screen® Q Fever Indirect Multi-species)

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

1. Samples are tested at a final dilution of 1:50 as follows:
    a. In a 96-well pre-dilution microplate, add:
        i. 5µl of the Negative Control to wells A1 and B1;
        ii. 5µl of the Positive Control to wells C1 and D1;
        iii. 5µl of each sample in the remaining wells;
        iv. 245µl of the Dilution Buffer 2 to each well.
2. In the ELISA microplate, transfer:
    a. 100µl of prediluted Negative Control to wells A1 and B1;
    b. 100µl of prediluted Positive Control to wells C1 and D1;
    c. 100µl of each prediluted sample in the remaining wells.
3. Cover the plate and incubate 45min (± 4min) at 21ºC (± 5ºC).
4. Empty the wells. Wash each well 3 times with 300µl of Wash Solution. Avoid drying of the wells between washes.
5. Prepare the Conjugate 1X by diluting the Concentrated Conjugate 10X to 1:10 in Dilution Buffer 3.
6. Add 100µl of the Conjugate 1X to each well.
7. Cover the plate and incubate 30min (± 3min) at 21ºC (± 5ºC).
8. Empty the wells. Wash each well 3 times with 300µl of wash solution. Avoid drying of the wells between washes.
9. Add 100µl of the Substrate to each well.
10. Cover the plate and incubate 15min (± 2min) at 21ºC (± 5ºC) in the dark.
11. Add 100 µl of the Stop Solution to each well.
12. Read and record the OD at 450nm.

Annex VI – DNA extraction kit (Qiagen® QIAamp DNA Mini Kit)

1. In an Eppendorf® tube add:
    a) 200µl of PBS homogenate of swabs or 25mg of tissues homogenates;
    b) 180µl of ATL Buffer;
    c) 20µl of Proteinase K.
2. Vortex for one minute.
3. Incubate at 70ºC for 30 minutes.
4. Vortex for a few seconds.
5. Add 200µl of Buffer AL and vortex for 15 seconds.
6. Incubate 10 minutes at 70ºC.
7. Add 200µl of 100% ethanol.
8. Vortex for 15 seconds.
9. Identify the columns and transfer the Eppendorf® content to the columns.
10. Centrifuge for one minute at 15000g.
11. Discard the collection tube and conserve the column by placing it in a new collection tube.
12. Add 500µl of Buffer AW1.
13. Centrifuge for one minute at 15000g.
14. Discard the collection tube and conserve the column by placing it in a new collection tube.
15. Add 500µl of Buffer AW2.
16. Centrifuge for one minute at 15000g.
17. Discard the collection tube and conserve the column by placing it in a new collection tube.
18. Centrifuge for three minutes at 15000g.
19. Discard the collection tube and place the column in a 1.5ml Eppendorf® tube.
20. Add 200µl of Buffer AE to elute the DNA. Allow to incubate for one minute at room temperature and centrifuge at 6000g for one minute.
21. Store specimens at refrigeration temperature if used immediately or freeze at -20ºC or -80ºC if analysis is later.

Annex VII – Real time PCR kit (Lifetechnologies® commercial Taq-Vet™ *Coxiella burnetii* kit)

The “Mix FQP” is ready to be used.

1. Vortex the tube containing the «Mix FQP» and pipette 20µl of «Mix FQP» for each sample to be tested in an Eppendorf® tube. Don’t forget the positive and negative controls.
2. On the equipment, create a microplate plan as follows:
    a) Use “ROX” as a passive reference and create two detectors – FQ (*reporter* FAM, *quencher* TAMRA) and IPC (*reporter* VIC, *quencher* TAMRA);
    b) Assign the FQ detector and the IPC detector to each well.
3. Dispense 20µl of mix per test sample onto the microplate for real-time PCR. In the Negative Control well, place 25µl of Mix.
4. Add the corresponding sample to each microplate well:
    a) External Positive Control: place 5µl of standard DNA (concentration 10^4 in qualitive tests and concentration 10^1 to 10^4 in quantitative tests);
    b) Samples: 5µl of DNA extracted from the sample.
5. Cover the plate and place in the thermal cycler with the following program:
    a) Step 1: 50°C – 2 minutes;
    b) Step 2: 95°C – 10 minutes;
    c) Step 3: 95°C – 15 seconds and then 60°C – 1 minute – 45 repetitions.

Annex VIII – Report of practical internship activities

ESCOLA UNIVERSITÁRIA VASCODAGAMA

MEDICINA VETERINÁRIA

REGISTO DE CASUÍSTICA

Nome aluno (a): Suzi Marina Alves Neves

Local (ais) de estágio : Hospital Veterinário Universitário de Coimbra

Período estágio : 01/10/2020 a 31/03/2021

Breve contextualização do EC: Estágio curricular realizado no âmbito de obtenção do grau de mestre em Medicina Veterinária, na área de ciências clínicas, dentro dos objetivos estipulados de acordo com o Regulamento do Estágio Curricular.

Casos clínicos presenciados	Caninos	Felinos	Bovinos	Ovinos/ Caprinos	Suínos	Equinos	Aves	Coelhos/ Outros	TOTAL
Dermatologia	45	29	0	0	0	0	0	1	75
Otologia	57	31	0	0	0	0	0	0	88
Oftalmologia	17	26	0	0	0	0	0	0	43
Odontologia	18	7	0	0	0	0	0	1	26
Doenças Parasitárias	28	3	0	0	0	0	0	0	31
Doenças Infeciosas	21	42	0	0	0	0	0	1	64
Toxicologia	7	1	0	0	0	0	0	0	8
Gastroenterologia	33	11	0	0	0	0	0	2	46
Hepatologia	2	0	0	0	0	0	0	0	2
Nefrologia/Urologia	14	34	0	0	0	0	0	0	48
Ginecologia	9	0	0	0	0	0	0	0	9
Pneumologia	18	27	0	0	0	0	1	3	49
Cardiologia	13	2	0	0	0	0	0	0	15
Neurologia	24	3	0	0	0	0	0	0	27
Endocrinologia	35	29	0	0	0	0	0	0	64
Oncologia	5	4	0	0	0	0	0	0	9
Ortopedia	26	18	0	0	0	0	0	0	44
TOTAL	372	267	0	0	0	0	1	8	648

Cirurgias presenciadas	Caninos	Felinos	Bovinos	Ovinos/ Caprinos	Suínos	Equinos	Aves	Coelhos/ Outros	TOTAL
Orquiectomia	18	12	0	0	0	0	0	0	30
Ovariohisterectomia	29	26	0	0	0	0	0	1	56
Cesariana	7	0	0	0	0	0	0	0	7
Mastectomia	10	2	0	0	0	0	0	0	12
Nodulectomia	21	15	0	0	0	0	0	0	36
Vulvoplastia	1	0	0	0	0	0	0	0	1
Nefrectomia	2	0	0	0	0	0	0	0	2
Esplenectomia	17	0	0	0	0	0	0	0	17
Hepatectomia parcial	1	0	0	0	0	0	0	0	1
Gastrotomia	9	0	0	0	0	0	0	0	9
Gastropexia	3	0	0	0	0	0	0	0	3
Enterotomia	0	1	0	0	0	0	0	0	1
Enterectomia	12	2	0	0	0	0	0	0	14
Hemilaminectomia	5	0	0	0	0	0	0	0	5

Artroscopia	6	3	0	0	0	0	0	0	9
TPLO	9	2	0	0	0	0	0	0	11
Resolução de fraturas ósseas	5	13	0	0	0	0	0	0	18
Ligamentoplastia	1	0	0	0	0	0	0	0	1
Caudectomia	1	0	0	0	0	0	0	0	1
Correção de proptose ocular	2	1	0	0	0	0	0	0	3
Reconstrução palpebral	0	1	0	0	0	0	0	0	1
Rinoplastia	0	1	0	0	0	0	0	1	2
Extirpação da glândula salivar	1	0	0	0	0	0	0	0	1
Destartarização	15	4	0	0	0	0	0	0	19
Lavagem broncoalveolar	0	2	0	0	0	0	0	0	2
Endoscopia	4	2	0	0	0	0	0	0	6
Ingluviotomia	0	0	0	0	0	0	2	0	2
TOTAL	179	87	0	0	0	0	2	2	270

Intervenções em sanidade e/ou produção animal	**Caninos**	**Felinos**	**Bovinos**	**Ovinos/ Caprinos**	**Suínos**	**Equinos**	**Aves**	**Coelhos/ Outros**	**TOTAL**
Vacinação	192	123	0	0	1	0	0	9	325
Desparasitação	257	142	0	0	1	0	0	13	413
Rastreio Sorológico	76	128	0	0	0	0	0	0	204
TOTAL	192	123	0	0	2	0	0	22	942

Necrópsias	**Caninos**	**Felinos**	**Bovinos**	**Ovinos/ Caprinos**	**Suínos**	**Equinos**	**Aves**	**Coelhos/ Outros**	**TOTAL**
	1	0	0	0	0	0	0	0	1
TOTAL	1	0	0	0	0	0	0	0	1