Universitat Autònoma de Barcelona

Epidemiological and clinicopathological study of Leptospira spp. infection in cats in Spain (Catalonia and Extremadura)

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Josep Pastor Milán, Associate Professor, and Rafaela Cuenca Valera, Professor of the Department of Animal Medicine and Surgery, Universitat Autònoma de Barcelona,

Inform:

That the report entitled **"Epidemiological and clinicopathological study of *Leptospira spp*. infection in cats in Spain (Catalonia and Extremadura)"**, presented by Diana Andrea Murillo Picco to obtain the degree of Doctor in Animal Medicine and Health from the Universitat Autònoma de Barcelona, has been carried out under our direction. Once it has been satisfactorily concluded, we authorize its presentation for evaluation by the corresponding committee.

And so that it may be recorded for the appropriate purposes, we signed the following report in Bellaterra on 10 July 2020.

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Dr. Rafaela Cuenca Valera
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to my mother Luz Marina Picco C.

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List of abbreviations

A/G	Albumin globulin ratio
AKI	Acute kidney injury
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
APPs	Acute phase proteins
Basos	Basophils
CBC	Complete blood count
CEEAH	Ethics Committee on Animal and Human Experimentation
CI	Confidence interval
CKD	Chronic kidney disease
CRP/Hp	C reactive protein-Haptoglobin ratio
DNA	Deoxyribonucleic acid
e.g.	Latin expression (*exempli grata*) meaning “for example”
ELISA	Enzyme-linked immunosorbent assay
Eos	Eosinophils
FeLV	Feline leukaemia virus
FIV	Feline immunodeficiency virus
GPox	Glutathione peroxidase
Hb	Haemoglobin
HCT	Haematocrit
HemO	Hemoxygenase
Hp	Haptoglobin
i.e.	Latin expression *(in exempla)* “that is”
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL1	Interleukin
IQR	Interquartile ranges
IRIS	International Renal Interest Society
IV	Intravenous
K3-EDTA	Tripotassium ethylenediaminetetraacetic acid

LIP	Lipoprotein
LPHS	Leptospiral pulmonary haemorrhage syndrome
LPS	Lipopolysaccharides
Lymphos	Lymphocytes
MAT	Microscopic Agglutination Test
Mce	Mammalian cell entry
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
Monos	Monocytes
N/A	Not applicable
NP	Not performed
Oag	O antigen
OIE	World Organization for Animal Health
OmpA	Outer membrane protein A
OR	Odds ratio
PBS	Phosphate buffered saline
PC1	Principal component 1
PC2	Principal component 2
PCA	Principal component analysis
PCR	Polymerase chain reaction
pH	Hydrogen potential
PLT	Platelets
pNA	p-nitrophenyl acetate
PO	Oral administration pathway
PON1	Paraoxonase-1
RBC	Red blood cells
RI	Reference interval
ROS	Reactive oxygen species
SAA	Serum amyloid A
SD	Standard deviation
Segs	Segmented neutrophils
SOD	Superoxide dismutase
St	Saint

TAC	Total antioxidant capacity
TEAC1	Trolox equivalent antioxidant capacity
TP	Total proteins
UPC	Urine protein creatinine ratio
USA	United States of America
USG	Urine specific gravity
WBC	White blood cells
WHO	World health organization
y.o.	Years old

Summary

Leptospirosis is a re-emerging zoonosis, caused by a bacterium of the genus *Leptospira*. It is the most widespread bacterial zoonosis worldwide, becoming a neglected disease in some regions. Its main reservoir are mammals, including domestic cats. It is usually spread through contaminated soil and water. An infected animal may develop an acute state of the disease or become a reservoir host. Research carried out during the last 8 years, has studied the cat as a reservoir of the infection. Cats may have an important role as leptospires carriers, contributing to their maintenance in the environment and favouring the zoonotic risk. Currently, there are no data on the epidemiology of the infection in cats in Spain and prevalence may vary depending on the geographical location, as it has been published worldwide.

Most of the clinicopathological data published associated with leptospirosis in cats are from acute or experimental infections. Nevertheless, the information in the species is very scarce in case of chronic renal carrier state of *Leptospira spp*. The differences in clinicopathological parameters between naturally infected and leptospires-free animals may assist veterinary clinicians in the recognition and diagnosis of chronic renal carrier state of *Leptospira spp*. in cats.

The general goal of this thesis was to provide more extensive knowledge about *Leptospira spp.* infection in cats, through the following specific objectives:

1. To evaluate the presence of antibodies against pathogenic *Leptospira* species and to determine the presence of *Leptospira spp.* DNA in urine and blood in free-roaming cats from two different geographical areas in Spain

2. To determine differences in haematology, biochemical profile and urinary parameters between naturally infected cats with *Leptospira spp.* and leptospires-free cats.

3. To assess the variables of the inflammatory response and antioxidant state in free-roaming cats naturally infected with pathogenic leptospires and leptospires-free cats.

Three studies were carried out. In the first one, we determined anti-leptospiral antibodies, blood DNA, and shedding of DNA from pathogenic *Leptospira* species in the urine of free- roaming cats, in two geographical regions in Spain. Anti-leptospiral antibodies were detected in 10/244 cats (4.1%). Titres ranged from 1:20 to 1:320 and the most common serovar detected was Cynopteri. Blood samples from 1/89 cats amplified for pathogenic *Leptospira spp.* DNA (1.12%). Urine samples from 4/232 cats amplified for leptospiral DNA (1.72%). In conclusion, free-roaming cats in Spain can shed pathogenic *Leptospira spp*. DNA in their urine and may be a source of human infection.

In the second study, the differences in haematology, biochemical profile and urinary parameters between naturally infected by pathogenic *Leptospira spp.* and leptospires-free cats were studied. Cats naturally infected by pathogenic leptospires had lower values of RBC, haemoglobin, albumin, creatinine and urea compared to leptospires-free cats. Positive *Leptospira spp.* DNA amplification cats were at high risk for the development of non-regenerative anaemias when compared with leptospires- free cats. In contrast, seropositive cats were more likely to have proteinuria when compared with leptospires-free cats. Chronic

infection and exposure to leptospires lead to haematological abnormalities and slight alterations in the biochemical profile and urinalysis.

The third study evaluated the inflammatory proteins (APPs) and the antioxidant response to gain knowledge about the course of the disease in cats, through a Principal Component Analysis (PCA). Our work concludes that *Leptospira spp.* DNA infected cats had an acute phase response, unlike, to seropositive infected cats. Besides, there was an increase in TAC serum concentrations indicating an antioxidant response in the infection, which is proportional to the antibody titre and not to the presence of bacterial DNA.

This thesis adds valuable information, with public health implication, of leptospirosis in cats and contributes to improving the knowledge of the disease, which remains poorly studied compared to dogs.

Resumen

La leptospirosis es una zoonosis re-emergente, causada por una bacteria del género *Leptospira*. Es la zoonosis bacteriana más extendida en todo el mundo, convirtiéndose en una enfermedad desatendida en algunas regiones. Su principal reservorio son los mamíferos, incluidos los gatos domésticos. Suele propagarse a través de suelos y aguas contaminadas. Un animal infectado puede desarrollar un estado agudo de la enfermedad o convertirse en un huésped reservorio. Las investigaciones realizadas durante los últimos 8 años han estudiado al gato como reservorio de la infección. Los gatos pueden tener un papel importante como portadores de leptospiras, contribuyendo a su mantenimiento en el ambiente y favoreciendo el riesgo zoonótico. Actualmente, no existen datos sobre la epidemiología de la infección en los gatos en España y es posible que la prevalencia varíe según la ubicación geográfica, tal y como se ha publicado a nivel mundial.

La mayor parte de los datos clínico-patológicos publicados relacionados con la leptospirosis en gatos, provienen de infecciones agudas o experimentales. Sin embargo, la información en la especie es escasa en el caso del estado de portador renal crónico de *Leptospira spp*. Las diferencias en los parámetros clínico-patológicos entre animales infectados de forma natural y no infectados, puedan ayudar a los veterinarios clínicos en el reconocimiento y diagnóstico de portadores renales crónicos de *Leptospira spp.* en gatos.

El objetivo general de esta tesis fue proporcionar un conocimiento más amplio sobre la infección por *Leptospira spp.* en los gatos, por medio de los siguientes objetivos específicos:

1. Evaluar la presencia de anticuerpos frente a especies patógenas de *Leptospira* y determinar la presencia de ADN de *Leptospira spp.*, en la orina y la sangre, en gatos de vida libre de dos áreas geográficas diferentes de España.

2. Determinar las diferencias en los parámetros hematológicos, bioquímicos y del uroanálisis, entre gatos infectados de forma natural por *Leptospira spp* y gatos libres de la infección.

3. Valorar las variables de la respuesta inflamatoria y las del estado antioxidante en gatos de vida libre infectados de forma natural por *Leptospira spp* y gatos libres de la infección.

Se realizaron tres estudios. En el primero, determinamos la prevalencia de anticuerpos anti-leptospirales, la presencia de ADN de *Leptospira spp.* en sangre y orina, en gatos de vida libre en dos regiones geográficas de España. Se detectaron anticuerpos anti-leptospirales en 10/244 gatos (4,1%). Los títulos de anticuerpos variaron entre 1:20 a 1:320 y el serovar encontrado con más frecuencia fue Cynopteri. Muestras de sangre de 1/89 gatos (1,12%) y muestras de orina de 4/232 (1,72%) gatos, amplificaron para la presencia de ADN de *Leptospira spp*. En conclusión, los gatos de vida libre en España pueden eliminar en su orina, ADN de leptospiras patógenas y ser una posible fuente para la infección humana.

En el segundo estudio, se evaluaron las diferencias en la hematología, el perfil bioquímico y los parámetros urinarios entre los gatos infectados de forma natural por *Leptospira spp.* y los gatos libres de ellas. Los gatos infectados por leptospiras patógenas tuvieron valores más bajos de eritrocitos, hemoglobina, albúmina, creatinina y urea, comparados con los gatos libres de leptospiras. Los

gatos positivos que amplificaron para el ADN de las especies de *Leptospira* patógenas mediante PCR (orina o sangre), corrían un riesgo mayor de desarrollar anemia no regenerativa, mientras que, en los gatos seropositivos era más probable que se produjera proteinuria. La infección crónica y la exposición a las leptospiras conduce a anormalidades hematológicas y a alteraciones ligeras en el perfil bioquímico y el uroanálisis.

En el tercer estudio se evaluaron a través de un Análisis de Componentes Principales (ACP), las proteínas inflamatorias (PFA) y la respuesta antioxidante para obtener un mayor conocimiento sobre el curso de la enfermedad en los gatos. Nuestro trabajo concluyó que los gatos infectados por el ADN de *Leptospira spp*. tienen una respuesta inflamatoria de fase aguda, a diferencia de los gatos infectados seropositivos. Además, hubo un aumento en las concentraciones séricas de CAT que indicaría una respuesta antioxidante en esta infección, que es proporcional al título de anticuerpos y no a la presencia de ADN bacteriano.

Esta tesis añade información valiosa con implicaciones para la salud pública, sobre la leptospirosis en gatos y contribuye a mejorar el conocimiento de la enfermedad, que sigue siendo poco estudiada en comparación con los perros.

Justification

Leptospirosis is the most neglected widespread zoonosis worldwide. The vast majority of mammals are hosts of *Leptospira spp.* Worldwide, 1.03 million cases of *Leptospira spp.* infections in humans have been estimated per year, of which 58,900 correspond to deaths. Cats can get infected with *Leptospira spp.* and become an acute or chronic host, although the clinical presentation of the disease is rare and usually mild. Cats develop specific antibodies against *Leptospira spp*., with no clear association with clinical disease and shed *Leptospira spp.* DNA in urine, with a prevalence depending on the geographical area, the presence in the area of farm animals infected with leptospires, and prey habits. Cats may have an important role as leptospires reservoirs, contributing to their maintenance in the environment and favouring the zoonotic risk.

Knowledge of the involved leptospiral serovars in animals from any country is imperative for the accurate diagnostics and epidemiological surveillance of the disease. Therefore, it is necessary a broader understanding of the infection seroprevalence, endemic serovars/serogroups and pathogenic *Leptospira spp.* DNA urinary shedding prevalence in cats in Spain.

In those diseases where the symptoms clinically are not too evident, the clinical-pathological data can help to understand more the course of the infection and thus to establish the diagnosis. In feline leptospirosis, clinical signs are, at best, mild and animals often are sub-diagnosed becoming in chronic renal carriers. Published reports on haematology values, biochemistry screen or urinalysis data associated with *Leptospira spp.* infection in cats are scarce and most of them from acute or experimental infections. Similarly, currently published information

on acute phase proteins and antioxidant biomarkers (which are likely to become an essential component to understand the course of infection, improving the diagnosis) in feline leptospirosis is nil. Accordingly, more information, that would provide valuable insights for clinicians in diagnosing chronic renal carrier state of *Leptospira spp*. in cats, is needed.

This thesis is organized in the following sections:

1. GENERAL INTRODUCTION, presented as a review article "LEPTOSPIROSIS IN CATS: Current literature review to guide diagnoses and management". *J. Feline Med. Surg.* 2020, 22, 216-228. https://doi.org/10.1177/1098612X20903601

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4.2. STUDY II: "Clinicopathological findings in cats naturally infected by *Leptospira spp."*, written for submission to the *Vet Clin Pathol* journal.

4.3. STUDY III: "Acute phase proteins and total antioxidant capacity in free-roaming cats infected by pathogenic leptospires" submitted to *BMC Vet Res* journal.

5. GENERAL DISCUSSION

6. CONCLUSIONS

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1. GENERAL INTRODUCTION

LEPTOSPIROSIS IN CATS: Current literature review to guide diagnosis and management

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Global importance: Leptospirosis is the most widespread zoonosis worldwide. Mammals (eg, rats, horses, cows, pigs, dogs, cats and aquatic species, such as sea lions and northern elephant seals) can all be infected by leptospires. Infection in animals occurs through contact with urine or water contaminated with the bacteria. In people, the disease is acquired mainly from animal sources or through recreational activities in contaminated water.

Practical relevance: Literature on the clinical presentation of leptospirosis in cats is scarce, although it has been demonstrated that cats are susceptible to infection and are capable of developing antibodies. The prevalence of anti-leptospiral antibodies in cats varies from 4% to 33.3% depending on the geographical location. Urinary shedding of leptospires in naturally infected cats has been reported, with a prevalence of up to 68%. Infection in cats has been associated with the consumption of infected prey, especially rodents. Thus, outdoor cats have a higher risk of becoming infected.

Clinical challenges: Clinical presentation of this disease in cats is rare and it is not known what role cats have in the transmission of leptospirosis. Ongoing work is needed to characterise feline leptospirosis.

Audience: This review is aimed at all veterinarians, both general practitioners who deal with cats on a daily basis in private practice, as well as feline practitioners since both groups face the challenge of diagnosing and treating infectious and zoonotic diseases.

Evidence base: The current literature on leptospirosis in cats is reviewed. To date, few case reports have been published in the field, and information has mostly been extrapolated from infections in people and dogs. This review is

expected to serve as a guide for the diagnosis and management of the disease in cats.

Keywords: Leptospirosis; microscopic agglutination test; real-time PCR; zoon

Aetiology

Leptospirosis is caused by spirochetal bacteria of the genus *Leptospira.* These are highly motile, elongated and helically coiled bacteria that differ morphologically from other spirochetes by having a ‘question mark’ or hook-shaped end.[1–3] The genus *Leptospira* was originally divided into two species: *Leptospira interrogans*, containing the pathogenic serovars, and *Leptospira biflexa*, containing the non-pathogenic saprophytic serovars.[4] However, this phenotypic classification has been largely superseded by genetic classification, based on genotypic identification techniques, that includes all serovars of *L. interrogans* sensu lato and *L. biflexa* sensu lato (sensu lato is a Latin phrase meaning ‘in the broad sense’ and is often used taxonomically to indicate a species complex).[3]

Currently, 22 species of *Leptospira* have been identified;[4] at least 10 of these are pathogenic. There are also seven saprophytic species and five species of indeterminate pathogenicity.[5] It is likely that more species will be described in the future. Pathogenic *Leptospira* species are divided into serovars, each with distinct antigenic compositions; to date, over 260 pathogenic serovars, arranged into 26 serogroups, have been identified. This serological classification, based on determining antigenic characteristics, is more useful diagnostically and also better serves epidemiological purposes.

All mammals may be susceptible to *Leptospira* infection.[3] There are primary (definitive) or carrier hosts for some serovars (eg, dogs are hosts for Canicola; cows and sheep for Hardjo; pigs for Pomona and Bratislava; and rats for Icterohaemorrhagiae and Copenhageni). These contribute to a greater extent to the spread of bacteria in the environment

compared with incidental or dead-end hosts (i.e. that suffer acute disease and are unlikely to serve as a source of transmission; eg, humans). The definitive host is typically infected at a young age and commonly exhibits minimal clinical disease, whereas animals infected with non-host-adapted serovars are expected to exhibit more severe clinical signs.[3]

Epidemiology

Leptospirosis is endemic in almost all regions of the world.[2] Its incidence usually increases at the end of the summer months, while in the tropics most infections occur during and after periods of rainfall.[1,2] Pathogenic *Leptospira* species experience optimal growth at temperatures of 28–30ºC. Although they do not replicate outside of the host, they can survive for months in moist soil saturated with urine,[1,3] and this can lead to significant environmental contamination. In people, there are three main factors associated with the risk of disease transmission: (1) water exposure; (2) exposure to carrier rodents; and (3) transmission from livestock or pets.[6]

Feline leptospirosis was first described in 1972,[7] and prevalence studies show the main serovars belong to serogroups Australis, Autumnalis, Canicola and

Sejroe,[8–18] although there are geographical variations. The most frequent serovars involved in feline leptospirosis in Europe – according to the European consensus statement on leptospirosis, and based on the prevalence of antibodies measured by the microscopic agglutination test (MAT) – belong to serogroups Australis, Autumnalis, Ballum, Canicola, Grippotyphosa, Icterohaemorrhagiae, Pomona and Sejroe.[19] The most commonly reported serovars in cats in the USA belong to serogroups Australis, Autumnalis, Grippotyphosa and Pomona.[8,20]

Figure 1 shows the countries where the prevalence of leptospirosis in cats has been reported, based on MAT and/or urinary and blood PCR. Table 1 summarises previous research on feline leptospirosis prevalence by MAT diagnosis. Overall seropositivity reported in these studies ranged from 4% to 33.3%, with no clear association with clinical disease. Leptospiral infection in cats has been associated with the consumption of infected prey,[29] involving serovars of the Autumnalis and Ballum serogroups.[3] Outdoor cats have an increased risk of becoming infected with leptospires since they are in close contact with reservoir hosts. In rural areas, cats can also become infected via urine from pigs and cows.[12,15,28–30] The presence of another cat in the household significantly increases the risk of seropositivity for leptospirosis.[14] At present, it is not completely understood which serovars cause incidental infections in cats. Based on previously published reports of acute leptospirosis in cats, serovars belonging to Autumnalis, Australis, Icterohaemorrhagiae, Grippotyphosa, Pomona and Sejroe serogroups are involved.[14,18,30–32] Several studies have confirmed renal carriage of *Leptospira* species by PCR, and these cats had antibodies mainly against serovars belonging to Australis, Canicola, Icterohaemorrhagiae and

Pomona serogroups. Given this fact, cats could be a chronic reservoir host for the bacteria and a possible risk factor for human infection.[10,11,13,14,16,26,30,33,3]

Figure 1 Map indicating (in red) the countries where the prevalence of leptospirosis in cats has been reported, based on microscopic agglutination test (MAT) and/or urinary and blood PCR.

Table 2 summarises the scant research that has been carried out in cats in different countries to determine the prevalence of *Leptospira* DNA shedding in urine. In these studies, the prevalence ranged from 0% to 67.8%, with no clear association with clinical disease. The prevalence may differ depending on the geographical location and the PCR-selected primers, among other factors.

Table 1. Prevalence data for leptospirosis in cats diagnosed by microscopic agglutination test

Location	*n*	Positive serovar(s)	Negative serovar(s)	Prev %	Ref
Malaysia – Johor and Selangor	110	Ballum, Bataviae*, Copenhageni and Javanica	Australis, Autumnalis, Canicola, Celledoni, Cynopteri, Djasiman, Grippotyphosa, Hardjo, Hardjobovis, Hebdomadis, Icterohaemorrhagiae, Lai, Malaysia, Pomona, Pyrogenes and Tarassovi	18.1	9
Thailand – 13 locations	260	Anhoa, Autumnalis, Celledoni, Copenhageni, Djasiman, Icterohaemorrhagiae and Patoc*	Australis, Ballum, Bataviae, Bratislava, Broomi, Canicola, Coxi, Cynopteri, Grippotyphosa, Haemolytica, Khorat, Paidjan, Pomona, Pyrogenes, Rachmati, Saxkoebing and Sejroe	5.4	10
USA – Iowa	139	Bratislava*, Grippotyphosa, Hardjo, Icterohaemorrhagiae and Pomona	Canicola	8.6	8
Germany – Munich	215	Australis*, Autumnalis, Bratislava, Copenhageni and Grippothyphosa	Canicola, Pomona and Saxkoebing	17.9	11
Caribbean island of St. Kitts	50	Cynopteri and Pomona	Alexi, Australis, Autumnalis, Bataviae, Ballum, Borincana, Bratislava, Canicola, Celledoni, Cynopteri, Djasiman, Georgia, Grippothyphosa, Hardjo, Icterhemorrhagiae, Javanica, Mankarso, Pomona, Pyrogenes, Tarassovi and Wolffi	4	21
Brazil – Lago Grande	43	Andaman and Patoc	Autumnalis, Australis, Bataviae, Bratislava, Butembo, Canicola, Castellonis, Copenhageni, Cynopteri, Grippotyphosa, Guaricura, Hardjo-prajitno, Hebdomadis Icterohaemorrhagiae, Javanica, Panama, Pomona, Pyrogenes, Shermani, Tarassovi, Whitcombi and Wolffi	4.7	22
Mexico – Merida	13	Australis and Canicola*	Autumnalis, Brastislava, Gryppotyphosa, Hardjo, Icterohaemorrhagiae, Pyrogenes, Panama, Pomona, Tarassovi and Wolff	23.2	23
Iran – Mashhad	147	Hardjo*, Icterohaemorrhagiae and Pomona	Autumnalis, Ballum, Canicola and Grippotyphosa	12.9	12
Taiwan – Southern Taiwan	225	Australis, Icterohaemorrhagiae, Javanica, Pyrogenes and Shermani*	Autumnalis, Bataviae, Canicola, Panama, Pomona and Tarassovi	9.3	13

Canada – Quebec	240	Bratislava, Grippothyphosa, Icterohaemorrhagiae and Pomona*	Canicola and Hardjo	10.8	14
Chile – Valdivia, Osorno, Paillaco y San Pablo	124	Autumnalis*, Bataviae and Canicola	Ballum, Hardjo, Icterohaemorragiae and Pomona	25.2	15
Serbia – Belgrade	161	Australis*, Bratislava, Canicola, Grippotyphosa Pomona* and Pyrogenes	Autumnalis, Batavie, Icterohaemorrhagiae and Sejroe	26.7	24
Canada – Quebec	40	Autumnalis and Bratislava*	Canicola, Grippotyphosa, Icterohaemorrhagiae and Pomona	25	25
Reunion Island	30	Panama	Australis, Autumnalis, Bataviae, Canicola, Castellonis, Cynopteri, Grippotyphosa, Hardjo-bovis, Hebdomadis, Icterohaemorrhagiae, Copenhageni, Pomona, Pyrogenes, Sejroe and Tarassovi	26.6	26
Iran – Ahvaz	102	Australis and Ballum*	Canicola, Grippotyphosa, Hardjo, icterohaemorrhagiae and Pomona	4.9	27
USA – Massachusetts	63	Autumnalis*, Bratislava, Icterohaemorrhagiae and Pomona	Canicola, Grippotyphosa and Hardjo	4.8	20
Spain – Andalucia	53	Ballum and Icterohaemorragiae*	Australis, Autumnalis, Bataviae, Bratislava, Canicola, Grippotyphosa, Hardjo, Hebdomadis, Pomona, Saxkoebing, Sejroe and Tarassovi	14	16
Greece – Thessaloniki	99	Ballum, Bataviae, Bratislava, Canicola, Panama, Pyrogenes and Rachmati*	Hebdomadis, Panama and Pomona	33.3	17
Scotland –Glasgow	87	Autumnalis, Hardjo-bovis* and Icterohaemorrhagiae	Autumnalis, Bratislava, Ballum, Canicola, Cynopteri, Grippotyphosa, Javanica, Pomona and Tarassovi	9.2	18
New Zealand –North Island	225	Balcanica, Ballum, Canicola, Copenhageni*, Hardjo* and Pomona	Australis, Bataviae, Javanica, Pyrogenes and Tarassovi	8.8	28

*Most prevalent serovar reported in the study, Prev = Prevalence

Table 2. Summary of current research on prevalence of urinary shedding of *Leptospira* DNA in cats

Location	*n*	Gene target/primer set	Prevalence %	Reference
Reunion Island	172	*rrs*2, *lip*L32 and *lip*L41	0.6	35
Thailand – 13 locations	260	*lip*L32	0.8	10
Algeria – Algiers	107	*rrs* (16S) and *hsp*	0	36
Germany –Munich	215	*lipL32*	3.30	11
Australia – Christmas Island	59	23S	42.4	33
Canada –Quebec	240	G1 and G2 and B64-I/B64-II primers	3.33	14
Reunion Island	30	*lipL32*	26.6	26
Taiwan – Southern Taiwan	225	G1/G2 and *Leptospira rrs* (16S)	67.8	13

Pathogenesis

Depending on the host and infecting serovar, leptospiral infection may cause spectrum of syndromes from asymptomatic carriage to fulminant, acute disease.[3] Reports of clinical disease due to *Leptospira* species in pet cats are scarce. Leptospires can enter the body through cuts and abrasions, mucous membranes, such as the conjunctiva, or through moist, weakened skin. The bacteraemia lasts around 7 days. The pathogenesis of the disease in cats remains unknown, although it is assumed to be similar to that in humans and dogs[37] (Figure 2). Acute clinical disease occurs with the bacteraemic phase of the disease.[1,2,38]

It is seen mainly in young incidental hosts and is usually associated with haemolysin producing bacteria, such as the Icterohaemorrhagiae or Pomona serogroups, which cause haemolytic disease, haemoglobinuria, jaundice and, in severe cases, death.[3] After leptospires have reached a critical level in the blood, clinical signs appear due to the action of leptospiral toxins or toxic cellular components.[1,2,38] Organ damage occurs as a result of leptospires replicating and inducing cytokine production and by direct invasion of inflammatory cells.[3]

The primary lesions develop in the endothelium of the small blood vessels, leading to localised ischaemia, and resulting in renal tubular necrosis, among other target organ damage (Figure 2). Renal colonization occurs in most infected animals because the bacteria replicate and persist in the cells of the renal tubule epithelium. This multiplication process causes the release of cytokines and the recruitment of inflammatory cells, which trigger nephritis.[1,2,38] Chronic interstitial nephritis, which may result in chronic renal damage, has been described in cats

infected with leptospires.[16] After 10 days of infection, leptospires enter the tubular lumen and are eliminated in the urine over a period of days to months.[1,2,38]
The duration of elimination via the urine and its intensity varies from species to species and animal to animal and depends on the infecting serovar;[3] precise information on these aspects is currently unavailable in cats.

As mentioned earlier, cats can act as carrier hosts, not developing clinical disease, but shedding bacteria into the environment in their urine. An epidemiological study has confirmed the presence of leptospiral DNA in the urine of cats for more than 8 months after infection, with little or no association with disease.[11] However, this does not rule out the possibility that infected animals could develop kidney disease at a later stage. The development of the carrier state and the specific mechanisms required for leptospires to enter the lumen of the proximal renal tubules, adhere to renal epithelial cells, evade antibodies in the filtrate and acquire the nutrients they need to replicate are not well understood.[3]

Leptospiral pulmonary haemorrhage syndrome (LPHS) has been recognised in people and dogs. This syndrome may be present in 70% of dogs infected with leptospires.[39] The clinical signs associated with canine LPHS are mainly acute and findings correspond to severe alveolar and subpleural haemorrhages, which cause an associated dyspnoea. While, to date, LPHS has not been described in cats, chronic liver inflammatory infiltration, fibrosis and multifocal hepatic necrosis have been reported.[14,16,31] Damage to organs including the spleen, eyes, meninges, muscle and placenta has also been reported in species other than cats.[2,3,40]

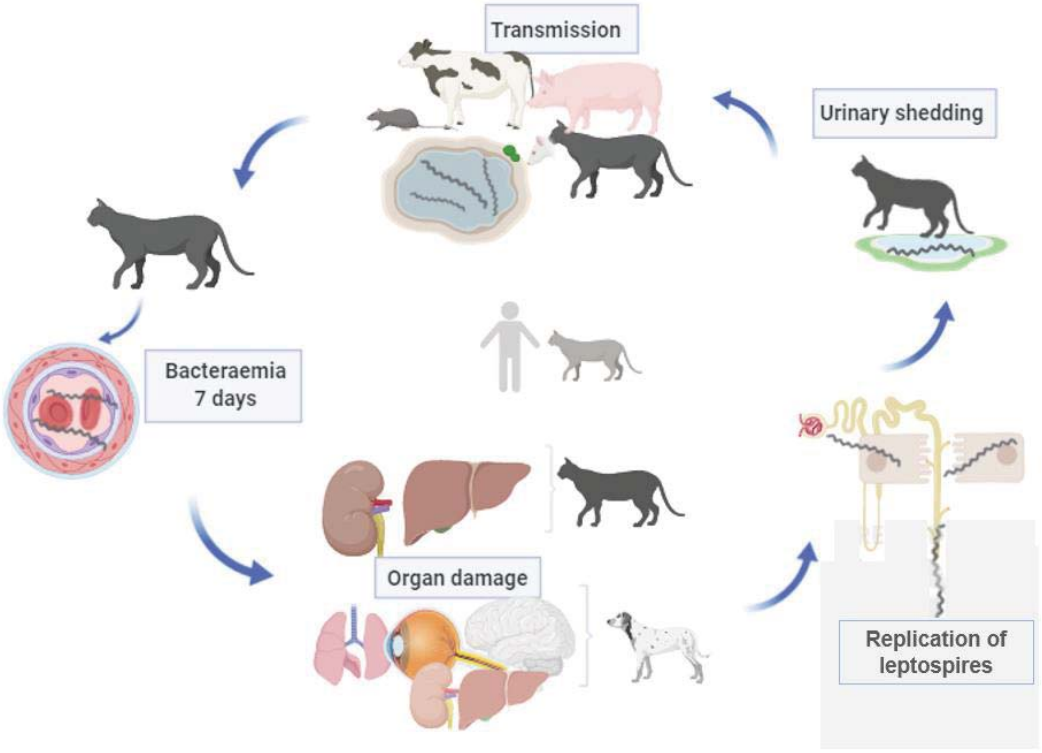

Figure 2 Proposed pathogenesis of leptospirosis in cats. The figure depicts the transmission mechanisms through which a cat can become infected by *Leptospira* species: preying on rodents, sharing the environment with farm animals that shed the bacteria in urine, or through standing water containing bacteria. Once the animal has become infected it suffers a period of bacteraemia of approximately 7 days and leptospires can be identified in blood. The main target organs in cats are the kidney and the liver; lungs, brain and eyes may also be affected, especially in dogs. Replication of leptospires occurs in the kidney leading to urinary bacterial shedding. *Image ©Biorender*

Virulence mechanisms and host factors

The virulence mechanisms of leptospires and the intrinsic factors of the host that determine the result of infection remain poorly understood. Recent mutagenesis studies in animal models of acute infection and of renal colonisation have

demonstrated that specific genes and proteins, among them lipoproteins LipL32 and LipL41 and LigB adhesin, are present in pathogenic *Leptospira* species, but are not necessary for virulence.[3,41,42] Loa22, an outer membrane protein containing a C-terminal OmpA domain, plays an indirect role in the virulence of *Leptospira* species.[43] Changes in motility through modifications in or mutations of genes involved in flagellar structure also play a role in the specific virulence of leptospires.[44,45] Adhesion to host tissues appears to be a prerequisite for successful infection; however, genetic studies have not confirmed a definitive role for many adhesins in the pathogenesis of *Leptospira* species.[3] With regard to the survival of leptospires in vivo, it has been suggested that pathogenic leptospires undergo receptor-mediated endocytosis[46,47] and are able to survive inside macrophages.[48] Production of pro-inflammatory cytokines and chemokines is more prolific in animal species susceptible to severe leptospirosis compared with resistant animal species.[49] Pathogenic leptospires are resistant to the bactericidal activity of complement, while saprophytic leptospires are highly susceptible.[41,50,51] The ability to acquire iron in vivo is a key virulence property for most bacterial species. Pathogenic leptospires possess heme oxygenase (HemO), which facilitates the acquisition of iron from heme, the major source of iron in the mammalian host.[52,53] However, no conclusive results were obtained in relation to attenuation of virulence of leptospires in a study using a HemO mutant.[54] Mutagenesis studies have also demonstrated that several stress response genes, which are upregulated with bacterial transition from the environment to the host, increase their susceptibility to oxidative stress and therefore render the bacteria less virulent.[55–57] In the same way, the inactivation

of Mce, a homologue of the mycobacterial mammalian cell entry protein in leptospires, has been found to result in a significant reduction in virulence.[58]

Diagnosis

Clinical signs

In cats, clinical signs are, at most, mild, despite the presence of leptospires in the blood and urine.

Clinical signs reported in infected cats (based on confirmation by MAT and/or PCR) include polyuria, polydipsia, haematuria, uveitis, lameness, lethargy, anorexia, weight loss, ascites, vomiting, diarrhoea, pain on handling, and inflammatory lesions on the skin and digits.[11,14,17,18,25,30–32,59] Pathological findings reported in these animals include the presence of haemorrhagic or straw-coloured thoracic and peritoneal fluids.[31,18] Some cats with antibodies against *Leptospira* species have been found to have signs associated with renal disease and/or histopathological evidence of renal inflammation.[14,16,30,35,59] As in dogs, leptospirosis in cats can cause acute kidney injury that leads to chronic kidney disease (CKD).[19,60] Lesions in the liver of affected cats have been reported less commonly than in dogs.[11,18,29,31,32]

Tables 3–5 collate information from several papers that detail the clinical signs in cats at the time of presentation, the laboratory test used for diagnosis and the *Leptospira* serovars involved. The cases have been divided into cats with acute disease (Table 3), those identified as chronic carriers (Table 4) and those with a history of exposure (Table 5).

Table 3. Clinical signs of leptospirosis in cats based on published studies of acute disease

	Reference	Serovar(s)	Clinical signs	Diagnosis	MAT	PCR
Prospective study	11	Australis	Seizures	Not reported	+	+
	11	Australis, Bratislava and Copenhageni	Acute diarrhoea	Not reported	+	+
	14	Bratislava, Grippotyphosa, Icterohaemorrhagiae and Pomona	Not reported	AKI	4 +	NP
	18	Autumnalis, Hardjo, and Icterohaemorrhagiae	Not reported	AKI	8 +	NP
Case report	32	Saxkoebing	Vomiting and diarrhoea, hyperaesthetic and painful on handling	AKI – Leptospirosis	+	+
	31	Grippotyphosa, Hardjo, Icterohaemorragiae and Pomona	Polyuria and polydipsia	AKI – Leptospirosis	+	NP
	31	Bratislava, Grippotyphosa and Pomona	Polyuria, polydipsia, haematuria and lameness	AKI – Leptospirosis	+	NP
	31	Autumnalis, Bratislava, Grippotyphosa and Pomona	Comatose	AKI – Leptospirosis	+	+
	30	Autumnalis And Pomona	Haematuria	Leptospirosis	+	+

MAT = microscopic agglutination test; + = positive; – = negative; CKD = chronic kidney disease; AKI = acute kidney injury; NP = not performed; 4+, 8+ = number of positive cats in the study

Table 4. Clinical signs of leptospirosis in cats according to published studies of chronic carrier cases

	Reference	Serovar	Clinical signs	Diagnosis	MAT	PCR
Prospective study	11	Australis and Bratislava	Asymptomatic	Incidental infection (Routine health check)	+	+
	11	Grippotyphosa	Not reported	Mast cell tumour in spleen and liver	+	+
	11	Not determined	Not reported	Foreign body in pharynx (grass)	-	+
	11	Grippotyphosa	Not reported	CKD and abdominal mass	+	+
	11	Australis, Autumnalis, Bratislava and Copenhageni	Chronic diarrhoea	Not reported	+	+
	14	Bratislava, Copenhageni Grippotyphosa and Pomona	Not reported	CKD	13 +	6 +
	14	Bratislava, Grippotyphosa, Icterohaemorragiae and Pomona	Not reported	Incidental infection (Routine health check)	9 +	2 +
	16	Icterohaemorragiae	Kidney**:** Chronic interstitial nephritis. Chronic inflammatory infiltration (macrophages and lymphocytes) Liver: Chronic inflammatory infiltration	Not reported	+	NP
	16	Canicola	Asymptomatic	Not reported	+	NP

MAT = microscopic agglutination test; + = positive; – = negative; CKD = chronic kidney disease; NP = not performed; 13+, 6+, etc = number of positive cats in the study

Table 5. Clinical signs of leptospirosis in cats based on published studies of animals with a history of exposure

	Reference	Serovar(s)	Clinical signs	Diagnosis	MAT	PCR
Prospective study	9	Ballum, Bataviae and Javanica	Not reported	Feline upper respiratory disease	+	NP
	15	Autumnalis, Bataviae, Canicola, and Grippotyphosa	Asymptomatic	Incidental infection (routine health check)	10 +	NP
	24	Australis, Bataviae, Bratislava, Canicola, Grippotyphosa, Icterohaemorrhagiae, Pyrogenes, Pomona and Sejroe	Asymptomatic	Incidental infection (routine health check)	43 +	NP
	25	Autumnalis and Bratislava	Not reported	CKD	+	NP
	25	Autumnalis and Bratislava	Polyuria and Polydipsia	Not reported	+	NP
	25	Autumnalis and Bratislava	Not reported	Hepatic lipidosis	2+	NP
	25	Autumnalis and Bratislava	Asymptomatic	Not reported	3+	NP
	27	Australis and Ballum	Asymptomatic	Not reported	5+	NP
	16	Icterohaemorrhagiae	Kidney**:** Chronic interstitial nephritis. Chronic inflammatory infiltrate (macrophages, lymphocytes and plasma cells). Proliferative glomerulonephritis. Liver**:** Multifocal hepatic necrosis. Chronic inflammatory infiltrate (lymphocytes and plasma cells)	Not reported	+	NP
	16	Ballum	Kidney: Chronic interstitial nephritis. Chronic inflammatory infiltrate (macrophages and plasma cells)	Not reported	+	NP

	16	Ballum	Asymptomatic	Not reported	+	NP
	16	Icterohaemorrhagiae	Kidney: Chronic interstitial nephritis. Chronic inflammatory infiltrate (macrophages and lymphocytes)	Not reported	+	NP
	17	Rachmati	Asymptomatic	Not reported	15+	NP
	17	Rachmati	Not reported	Various chronic diseases	18+	NP
Retrospective study	59	Bratislava and Icterohaemorrhagiae	Not reported	CKD – Azotaemia	4+	NP
	59	Bratislava and Icterohaemorrhagiae	Not reported	CKD – non-Azotaemia	8+	NP

MAT = microscopic agglutination test; + = positive; – = negative; CKD = chronic kidney disease; NP = not performed; 10+, 43+, etc = number of positive cats in the study.

Clinicopathological data

Table 6 summarises some of the most common clinicopathological abnormalities associated with leptospirosis in cats.

Complete blood count

Leukocytes can fluctuate according to the stage and severity of infection. Leukopenia is a possibility during leptospiraemia, evolving to leukocytosis owing to neutrophilia with a left shift. In advanced states, leukocyte counts may be in the range of 16.5–45 x 10^9/l (reference interval 2.75–11.75 x 10^9/l).[18,19,60,61]

Serum biochemistry

Urea and creatinine concentrations are increased in 80–90% of cases of canine leptospirosis.[60] Most infected cats present with azotaemia at the time of diagnosis. The increase is usually moderate to severe.[11,14,25,31,32,59] In affected dogs, serum liver enzyme (alkaline phosphatase [ALP] more commonly than alanine aminotransferase [ALT]) and total bilirubin increases are associated with liver dysfunction.[38,40,60] Conversely, in feline leptospirosis these increments are not as characteristic, and only slight increases have been reported.[11,18,25,31,32] Leptospires toxins inhibit Na+K+-ATPase activity in the epithelial cells of the renal tubules in cats and dogs, which can lead to significant renal losses of electrolytes, resulting in severe hypokalaemia.[60] In cats, increases in serum phosphorus concentration have been reported, probably associated with a decrease in the glomerular filtration rate.[31]

Urine analysis

Findings in dogs include isosthenuria or occasionally hyposthenuria, glycosuria, proteinuria, bilirubinuria, haematuria, pyuria and the presence of casts in fresh urine sediment.[38,60] In cats, hyposthenuria, haematuria and proteinuria have been

reported.[30,31] Leptospires are not visible on routine fresh urinary sediment examination, as the size of the bacteria is below the resolution of light microscopy.[19]

Table 6. Clinicopathological findings in 19 cats diagnosed with leptospirosis[11,18,30-32]

	Finding	Clinical course	Affected cats/ sampled cats
Haematology	Non-regenerative anaemia	Chronic carrier	2/19 (10.5%)
	Haemoconcentration	Acute disease	2/19 (10.5%)
	Neutropenia	Acute disease	1/19 (5.2%)
	Neutrophilia	Acute disease	2/19 (10.5%)
	Leucocytosis with left shift	Acute disease	2/19 (10.5%)
	Thrombocytopenia	Acute disease	1/19 (5.2%)
Biochemistry	Hypoalbuminaemia	Acute disease	2/2 (100%)
	Azotaemia	Acute disease and chronic carrier	9/19 (47.4%)
	Liver enzymes increased	Acute disease	6/8 (75%)
	Hyperglycaemia	Acute disease	1/2 (50%)
	Hyperphosphataemia	Acute disease	1/1 (100%)
	Bicarbonate decreased	Acute disease	1/1 (100%)
Urine analysis	Low USG	Acute disease and chronic carrier	3/5 (60%)
	Proteinuria	Acute disease	3/6 (50%)
	Haematuria	Acute disease	2/6 (33.3%)
	Renal and red cell cast	Acute disease	1/5 (20%)
Serological test	FeLV/FIV negative	Acute disease	5/5 (100%)

USG = urine specific gravity; FeLV = feline leukaemia virus; FIV = feline immunodeficiency virus

Ultrasonographic findings

The few published reports of feline leptospirosis describe renal ultrasonographic findings that are similar to those in canine leptospirosis, including a granular appearance of the kidney, enlarged kidneys with a cortex that is thinner than the medulla, a slightly hyperechogenic renal cortex and a decrease in the definition of the corticomedullary junction.[31,32] Heterogeneity in the pancreatic and liver parenchyma has also been reported in one case.[32]

Specific testing

Laboratory diagnosis of leptospirosis in veterinary medicine is usually based on the demonstration of serum antibodies by MAT and ELISA, and/or isolation of *Leptospira* DNA from blood and urine by PCR. Bacterial culture of blood and/or urine is not widely used because it is time consuming. Specific diagnostic tests that are available for cats are MAT and PCR.

Microscopic agglutination test

Determination of antibody titre by MAT is the recommended technique for leptospirosis diagnosis, as MAT reactivity to a serovar suggests exposure to a serovar belonging to the corresponding serogroup (though not necessarily to the specific serovar tested).[62] The selection of the serogroups and the serovars to be evaluated depends on the geographical location of the patient's likely exposure. Antibodies (IgM and IgG) are detected at around 15 days post-infection by MAT.[3] Little information is available on the duration of these antibodies in the blood of cats. Clinical interpretation should always be based on the results of paired serum titres, and it is worth noting that some infected animals may produce a result that is lower than the widely accepted minimum significant titre result of 1:100.[3] It is

even possible that seroconversion in cats is expressed at a lower titre compared with dogs.[59]

MAT results are strongly dependent on laboratory quality control, and there is considerable inter-laboratory variability.[63] Practitioners are encouraged to submit diagnostic samples to laboratories that adhere to a proficiency scheme.[64] Test interpretation may be more reliable in cats than in dogs because no interference with vaccine antibodies exists as cats are not vaccinated.[37] Furthermore, laboratory-reared young adult specific pathogen-free cats infected with *Borrelia burgdorferi* did not form antibodies against *Leptospira* species as a cross-reaction.[59] The authors of that study suggest that positive *Leptospira* species MAT results from cats in the field are likely to reflect antibodies against leptospires and not *B. burgdorferi*.

ELISA and rapid immunodiagnostic screening tests

ELISAs used for leptospirosis identify the presence of leptospiral antibodies (specific IgM class antibodies) earlier than MAT, at between 4–6 days post-infection.[3] The main advantages of ELISA compared with MAT are, in the authors' opinion, the stability of antigenic preparations and the genus specificity, meaning all types of leptospires can be diagnosed with a single antigenic preparation, irrespective of the causal serovar.[65] In dogs, a combination of ELISA plus MAT is recommended for leptospirosis diagnosis.[19]

Rapid patient-side tests for leptospirosis diagnosis were developed almost a decade ago.[66] Curtis et al performed a recombinant LipL32-based rapid in-clinic ELISA (SNAP Lepto) for the detection of antibodies against *Leptospira* species in dogs in 2015.[67] Neither of the tests distinguish between serovars, nor do they provide a titre magnitude. The first test[66] is based on the detection of *Leptospira*-

specific IgM and has demonstrated a sensitivity and specificity of 100% and 95.3%, respectively. It can therefore detect dogs with clinically suspected acute leptospirosis. Dogs previously vaccinated or suffering from an acute but subclinical infection can also produce positive results. A LipL32-based in-clinic ELISA for the rapid detection of *Leptospira* specific antibodies in dogs is not IgM specific, but the study authors considered it a convenient tool to assess *Leptospira* antibody status in dogs.[67]

Neither rapid test techniques, nor ELISA, to diagnose leptospirosis in cats have yet been developed.

PCR

PCR directly identifies leptospiral DNA. It does not determine the infecting serogroup or serovar, but it can indicate the *Leptospira* species. The test can be performed on blood, urine, cerebrospinal fluid and body tissues. In cases of acute leptospirosis, this would be the test of choice to perform on blood and urine in cats. Compared with culture, PCR gives fast results, contributing to an early diagnosis.[65] Real-time PCR techniques are recommended, due to their greater sensitivity and specificity. Genes that have more than one copy in the genome, such as *lig* or *rrs*, should be selected with the aim of increasing the sensitivity of the technique. Genes present only in the pathogenic species can also be added as they will increase the specificity of the test.[68]

A positive PCR result means that leptospiral DNA is present in the sample. In acute infections or in chronic carriers, the test would be positive in urine, indicating that bacterial DNA is being shed. However, negative results in blood and urine do not rule out leptospirosis, as leptospiraemia is transient (only occurring in the initial phases of the disease); also results are usually negative if

the cat has received antibiotic therapy,[19,60] and shedding in urine can be intermittent.[3] In one report, leptospires were cultured from cat urine and the results were confirmed by PCR,[34] suggesting that cats can shed living *Leptospira* bacteria, not just their DNA.

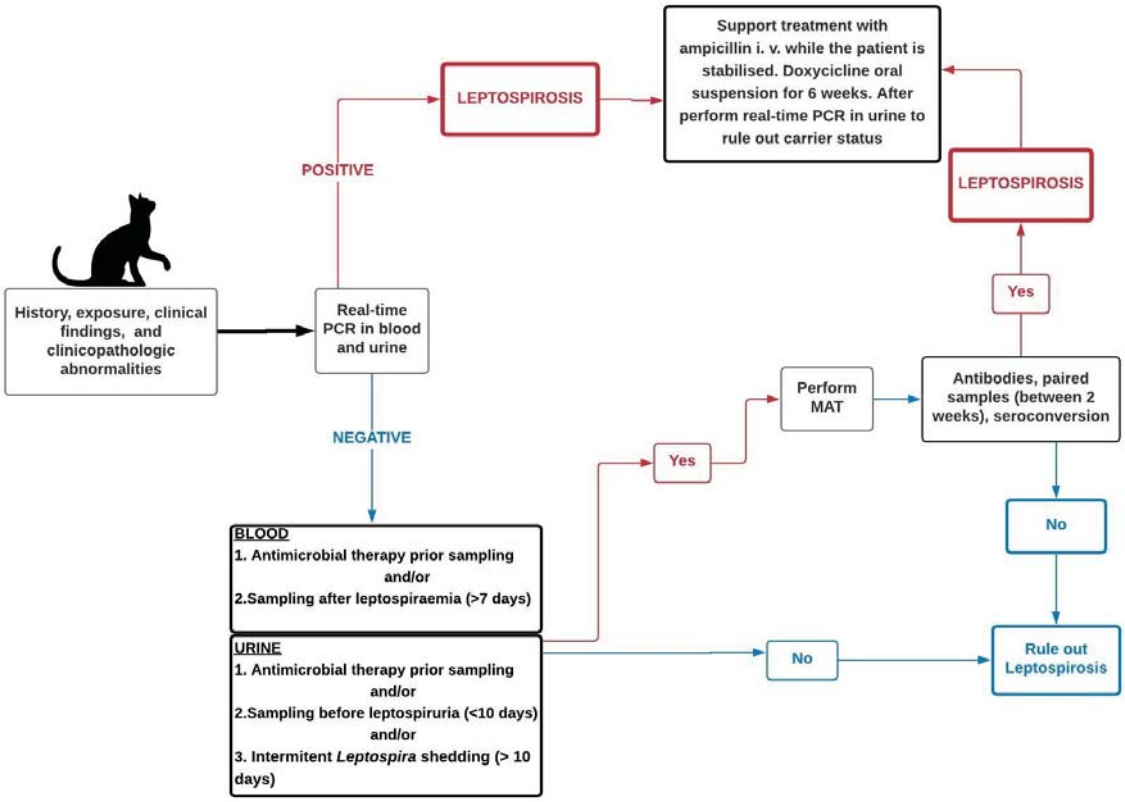

Figure 3 Diagnostic approach for a cat with suspected leptospirosis. MAT: microscopic agglutination test

Treatment

Supportive therapy

Intravenous fluids should be given to affected animals to correct the electrolyte fluid imbalance. The use of centrally acting antiemetics and the parenteral administration of gastric protectors is recommended in cats that develop

associated renal failure. Pain management is particularly important in the early stages of the disease to treat painful swollen kidneys, muscle, joints and gastro - intestinal tissue.[19]

Enteral feeding tubes are highly recommended in cats with anorexia, until they can feed themselves in a self-sufficient manner, minimizing the risk of secondary complications.[38]

Antimicrobial therapy

The antimicrobial therapy suggested in cats is based on the treatment recommended for dogs. Intravenous ampicillin may be the antibiotic of choice while the patient is stabilised. Once the animal is stable, 6 weeks of doxycycline oral suspension has been suggested in order to eliminate the carrier state.[37] Monohydrate salt of doxycycline, which is less irritating to the cat's oesophagus than hyclate or hydrochloride doxycycline salt, is marketed as tablets or suspension. Doxycycline monohydrate tablets should be administered immediately before a meal or with a treat in order to avoid secondary oesophagitis.[69,70]

Prognosis

The prognosis depends principally on the severity of any organ damage. In dogs that develop LPHS, the prognosis is usually poor since the animal is in acute respiratory failure with associated dyspnoea resulting from damage to the small pulmonary vessels, as described earlier; LPHS is one of the most common leptospiral-associated causes of death in dogs. The prognosis is also poor for

affected animals that develop acute renal injury unless haemodialysis is available.[60]

In cats with mild clinical signs and no severe organ damage, response to specific antimicrobial therapy and outcome are good.[30–32] Cats that survive acute renal failure, and especially those treated in the chronic phase of leptospirosis, may develop renal damage as a consequence of the initial condition, and this may be permanent.

Prevention

There is no commercial vaccine available for cats. However, one study has shown that cats can produce antibodies (of lower titre magnitude than vaccinated dogs) when experimentally inoculated with a commercial dog vaccine (containing four different serovars).[59] The follow-up time for the animals was 42 days, at which point only one animal maintained antibody levels. The authors of that study suggest further work is needed before a vaccine against *Leptospira* species for cats can be considered.

Given the current lack of a vaccine, the best way to avoid infection in cats is via prevention of exposure. Cats that are kept indoors have a lower risk of being infected.[37] Prevention of predation opportunities and avoidance of contact with stagnant water, urine from infected animals and dogs at risk of clinical leptospirosis is recommended.[6,30,31,37] For cats that share an environment with a positively diagnosed animal, doxycycline can be given at 5 mg/kg PO q12h or at 10 mg/kg PO q24h for 2 weeks.[19,60]

Case notes

A 2-year-old intact male domestic shorthair cat, showing lethargy and anorexia for 5 days, was presented for investigation.

Case work-up. The cat was an indoor-outdoor animal with a history of hunting small rodents. Vaccinations were current. On admission, the cat was quiet and depressed. On physical examination, body temperature was 39.7ºC and the cat's mucous membranes were pale and dry.

figure 4 Patient diagnosed with leptospirosis. Courtesy of Fundaciò Hospital Clínic Veterinari, Universitat Autònoma de Barcelona (UAB), Spain.

Heart rate was 180 beats per minute and pulse strength was normal; no murmurs or gallop rhythms were detected. Clinical dehydration was estimated to be 7%. There were no other remarkable findings upon physical examination. Owing to the non-specific clinical signs, blood samples were collected for haematology, biochemistry and feline leukaemia virus (FeLV)/feline immunodeficiency virus (FIV) testing. A urine sample was obtained by cystocentesis for complete urine analysis. Clinical pathology results are shown in the table. Mild leukocytosis with mature neutrophilia and thrombocytopenia were observed. Serum biochemistry profile showed a mild uraemia, increase in ALT and hyperproteinaemia (mainly due to an increase in the globulin fraction).

Table 7 Clinical pathology results

Complete blood cell count		
Analyte	**Result**	**RI**
RBCs (x 10^{12} /l)	6.2	6-10.2
Hb (g/dl)	10.5	9-15
HCT (l/l)	0.3	0.3-0.5
MCV (fl)	47.2	41-53
MCHC (g/dL)	36	30-34
Leucocyte (x10^9/l)	18.2	5.0-15.0
Lymphocytes (x10^9/l)	5.8	1.4-6.1
Monocytes (x10^9/l)	1.8	0.1-0.6
Band neutrophils (x10^9/l)	0	0-300
Segmented neutrophils (x10^9/l)	12.0	2.5-11.3
Platelet (x 10^9/l)*	115	200-600
Serum biochemistry (selected data)		
	Result	**RI**
Albumin (g/l)	24.8	23-34
Globulins (g/l)	44.0	26-38
Total Proteins (g/l)	96.9	54-78
Creatinine (µmol/l)	91	44.2-132.6
Urea (mmol/l)	8.6	3.32-8.3
ALT (UI/l)	51.4	< 50
Urine analysis (cystocentesis)		
	Result	
USG	>1.050	
pH	6	
Nitrite	Neg	
Protein	Neg	
Glucose	Neg	
Ketones	Neg	
Bilirubin	Neg	
Blood	Neg	
Sediment	No abnormalities identified Moderate fat droplets	
UPC	0.23	

*Only mild platelet aggregates were observed on blood smear, RBCs = red blood cells; Hb = haemoglobin; HCT = haematocrit; MCV = mean cell volume; MCHC = mean cell haemoglobin concentration; ALT = alanine aminotransferase; UPC = urine protein:creatinine ratio; RI = reference interval

FeLV antigen and FIV antibody testing were negative (IDEXX SNAP Combo Test). Most of the clinical pathological changes were suspected to be due to dehydration. However, thrombocytopenia and leukocytosis suggested a possible infectious origin. Ancillary tests, including an abdominal ultrasound examination and thoracic radiography, were performed. The results were unremarkable. Given the patient's predation habits, leptospirosis was considered and PCR (blood and urine) was performed. In addition, serum was serologically examined by MAT against eight *Leptospira* serovars: Australis, Autumnalis, Canicola, Grippotyphosa, Icterohaemorrhagiae, Javanica, Pomona and Sejroe. MAT serology for leptospirosis was negative.

Diagnosis. Blood PCR for leptospirosis was positive and urine PCR was negative.

Treatment and outcome. The cat was maintained on fluid therapy and antimicrobial therapy with ampicillin (20 mg/kg IV q12h) for 4 days. On day 4 of hospitalisation, ALT, creatinine and urea were re-checked; values were within reference intervals. On discharge, after receiving PCR blood results, doxycycline at 5 mg/kg PO q12h for 6 weeks was prescribed. Instructions were given to keep the animal confined during the next 6 weeks and isolated from other cats and dogs. The owners were advised to wear gloves while cleaning the litter box during this period, to use routine household disinfectants to clean the litter box and to wash their hands after handling their cat. Six weeks later, the cat had recovered completely, and no abnormalities were observed upon physical examination. A further urine PCR was recommended in order to rule out a chronic carrier state, as well as a further MAT to assess seroconversion, but for economic reasons this was not approved by the owners.

What this case demonstrates:

- While leptospirosis is an uncommon infectious disease in cats, practitioners should consider leptospirosis as a differential diagnosis in cats that hunt small rodents.
- At the time of admission, clinical pathological data in sick cats are not always characteristic of the disease, as in this case.
- For acute infections, PCR on blood and urine is the first-choice test to perform; MAT antibody titres are likely to be negative or low at that point, and seroconversion is not expected until 15 days post-infection.
- Leptospirosis is a zoonosis and special handling methods are needed in these cases: (1) avoiding contact with cats' urine and wearing gloves when cleaning the litter box; (2) using disinfectants to clean the cat's litter box as well as any other areas where the cat urinates; (3) preventing the cat from going outside to urinate in the environment; and (4) always washing hands after handling the cat.
- Owing to the zoonotic potential of leptospires, prophylactic treatment of other pets in the same household that may have been exposed to leptospires in the environment is recommended.
- When a diagnosis of leptospirosis is made, veterinarians should inform pet owners of the zoonotic risk of *Leptospira* bacteria and recommend medical attention if any family member develops signs of illness consistent with leptospirosis.

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2. HYPOTHESES

Leptospira spp. naturally, infection in cats has been recognized for more than a decade. Although the clinical presentation of leptospirosis in cats is scarce, cats may have an important role as leptospires reservoirs, contributing to their maintenance in the environment and favouring the zoonotic risk. Currently, there are no data on the epidemiology of the infection in cats in Spain. As the prevalence of anti-leptospiral antibodies varies depending on the geographical location and outdoor cats have a higher risk of getting infected, we believe it is more likely that cats from a geographic area with a high density of extensive livestock, could have a higher prevalence of anti-leptospiral antibodies, as well as a higher likelihood of having the bacteria in blood or urinary shedding, than cats from an urban geographic area.

In cats, clinical signs are, at most, mild and therefore the disease may be missed in the clinical practice. If the animals infected with *Leptospira spp.* had changes in their clinicopathological data as compared to those leptospires-free, this information can help to understand more the course of the infection and thus to improving the diagnosis, along with the diagnostic tests currently used for leptospirosis. Additionally, provide comprehensive knowledge on the chronic renal carrier state of *Leptospira spp*. in cats and valuable insights for clinicians for diagnosing.

There is a knowledge gap that needs to be addressed, bearing in mind the concept of “one health”, as cats can have significant zoonotic implications in the maintenance of the bacteria in the environment.

3. OBJECTIVES

Consequently, the general objective of this thesis was to provide a broader understanding of *Leptospira* spp. infection in cats, through the following objectives:

1. To evaluate the presence of antibodies against pathogenic *Leptospira* species and to determine the presence of *Leptospira spp.* DNA in urine and blood in free-roaming cats from two different geographical areas in Spain.

2. To determine differences in haematology, biochemical profile and urinary parameters between naturally infected cats with *Leptospira spp.* and leptospires-free cats.

3. To assess the variables of the inflammatory response and antioxidant biomarkers in free-roaming cats naturally infected with pathogenic leptospires and leptospires-free cats.

4. STUDIES

4.1. STUDY I

Leptospira Detection in Cats in Spain by Serology and Molecular Techniques

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Abstract

Leptospirosis is the most neglected widespread zoonosis worldwide. In Spain, leptospirosis reports in people and animals have increased lately. Cats can become infected with *Leptospira*, as well as be chronic carriers. The aim of this study was to determine serological antibody prevalence against *Leptospira* sp., blood DNA, and shedding of DNA from pathogenic *Leptospira* species in the urine of cats in Spain. Microagglutination tests (MAT) and blood and urine TaqMan real-time polymerase chain reaction (PCR) were performed. *Leptospira* antibodies were detected in 10/244 cats; with 4.1% positive results (95% confidence interval (CI): 2.1–7.18%). Titres ranged from 1:20 to 1:320 (serovars Ballum; Bataviae; Bratislava; Cynopteri; Grippotyphosa Mandemakers; Grippotyphosa Moskva; Pomona; and Proechimys). The most common serovar was Cynopteri. Blood samples from 1/89 cats amplified for *Leptospira* DNA (1.12%; 95% CI: 0.05–5.41%). Urine samples from 4/232 cats amplified for *Leptospira* DNA (1.72%; 95% CI: 0.55–4.10%). In conclusion free-roaming cats in Spain can shed pathogenic *Leptospira* DNA in their urine and may be a source of human infection. Serovars not previously described in cats in Spain were detected; suggesting the presence of at least 4 different species of pathogenic leptospires in the country (*L. borgpetersenii*; *L. interrogans*; *L. kirschneri*; *and L. noguchii*).

Keywords: antibodies; free-roaming cat; leptospirosis; PCR; shedding; serovar; zoonoses.

Introduction

Leptospirosis is a zoonosis present in every continent except Antarctica. The vast majority of mammals have been shown to be hosts of *Leptospira*[1,2]. Worldwide, 1.03 million cases of *Leptospira* infections in humans have been estimated per year, of which 58,900 correspond to deaths[3]. The incidence of human leptospirosis in Spain is 0.86 cases per million inhabitants. Catalonia and Extremadura are two of the autonomous communities with the highest reported cases[4,5]. It has been shown that some pathogenic species of leptospires like *Leptospira borgpetersenii*, *Leptospira interrogans*, *and Leptospira kirschneri* can naturally infect cats[6–8]. Clinical presentation of the disease is rare and usually mild in cats[9–12].

Several studies in different geographical areas have demonstrated that cats have contact with *Leptospira* since they develop specific antibodies ranging from 4% to 33.3%, with no clear association to clinical disease[13–17]. Urinary shedding of *Leptospira* DNA has also been documented in cats, with a prevalence ranging up to 67.8% depending on various factors including the geographical area, the presence in the area of farm animals infected with leptospires, and prey habits[7,14,15,18–21]. Furthermore, a recent study investigated the ability of cats to excrete viable bacteria through urine (p. 227,[19]), suggesting the possibility that cats can spread the bacteria by urine and infect humans. Sequences from bacterial DNA isolated in acute human cases of leptospirosis and wild animals have shown similarities with those isolated from cats in Reunion Island *(Leptospira borgpetersenii)*; the authors of the work rejected, however, any major role of feral cats in the epidemiology of leptospirosis in that geographical region due to urinary *Leptospira* shedding cats extremely low prevalence (0.6%)[8].

Conversely, other researchers argue that the role of cats in the maintenance of the pathogen has so far been underestimated[6,15,19]. It is possible that cats have an important role as leptospires carriers, contributing to their maintenance in the environment and favouring the zoonotic risk. The aim of this study was to evaluate the presence of antibodies against pathogenic *Leptospira* species and to determine the presence of *Leptospira* DNA in urine and blood by means of polymerase chain reaction (PCR), in free-roaming cats from two different geographical areas in Spain.

Materials and Methods

Sample size was determined based on the formula ($n = Z^2 \times P \times (1-P) / d^2$ where *n* = required sample size; *Z* = Z statistic for a level of confidence; *P* = expected prevalence or proportion based on literature in proportion of one; and *d* = precision in proportion of one), proposed for prevalence studies. Based on an assumed prevalence of antibodies against *Leptospira* species in cats of 17.9% and leptospiral DNA shedding in urine of 3.3%[15], a sample size of 226 cats (95% CI; 5% precision) was required for antibody prevalence and 194 cats (99.9% CI; 5% precision) for DNA urinary shedding study.

Animals in the Study

Animals from two different geographical regions of Spain (Barcelona, Catalonia, in the northeast and Cáceres, Extremadura, in the southwest) were used in this prospective trial. Two hundred and forty-four cats were recruited from October 2017 to September 2018. Cats from Barcelona (90/244) were part of a neutering program and were housed in local animal shelters in Barcelona; cats from Extremadura (154/244) were part of a free-roaming cat spay program in Cáceres.

Owing to the feral nature of most cats, prior history was not available. Data for gender, estimated age, and breed were collected. Signed informed consent was obtained from all animal shelters. Sampling collection was performed under the guidelines of the Ethical Committee Animal Care and Research, Autonomous University of Barcelona, approval number CEEAH, code 2939.

Sample Collection

In all cases, samples were collected with the cat under general anaesthesia. Three ml of venous jugular blood, collected from each animal, was divided and 1 ml was transferred to a K3-EDTA tube and 2 ml to a serum separator tube. K3-EDTA tubes were frozen at −20 °C until the DNA extraction. Serum separator tubes were centrifuged at 1300 × g, 5 minutes, within 5 hours of collection and the serum was stored at -20 °C until testing for leptospiral antibodies. Urine was collected by direct cystocentesis in sterile syringes and centrifuged at 14,000× g, 15 minutes at room temperature. The supernatant was discarded, and the pellet was re-suspended in a ratio of 1:1 with 0.5 ml Buffer phosphate-buffered saline (PBS) (pH 7.6, 0.01 M, Canvax, Córdoba, Spain). The final pellet volume (1ml) was stored at −20 °C until DNA extraction was performed. All cats were tested for feline leukaemia virus, FeLV and feline immunodeficiency virus, FIV, using a commercial test (SNAP Combo FeLV/FIV test®, IDEXX, Barcelona, Spain).

Microscopic Agglutination Test (MAT)

Microscopic agglutination test (MAT) was performed at the OIE and National Collaborating Centre for Reference and Research on Leptospirosis, Amsterdam, the Netherlands. MAT was performed by direct reading following a technique described before[22]. When a reaction was observed, it was re-checked by indirect

reading. Two-fold serial dilutions of serum from 1:20 to 1:160 were tested; this is including the antigen. In the case of animals with titres ≥1:160, the test was repeated, with a serial dilution series from 1:20 to 1:10240. Any antibody titre ≥1:20 was considered as a positive value[23]. Serum was examined for antibodies against 8 species of *Leptospira,* belonging to 20 serogroups, 27 serovars, and 28 strains (Table 1). Saprophytic strains *L. biflexa* strain Patoc I, *L. biflexa* strain CH 11, and *L. meyeri* strain Veldrat Semarang 173, were included in our panel diagnostic according to the World Health Organization's guidance[24].

Table 1. Species, serogroup, serovar, and strain from *Leptospira* tested among 244 cats from Spain.

SPECIES (8)	SEROGROUP (20)	SEROVAR (27)	STRAIN (28)
L. biflexa*	Andaman	Andaman	CH 11
L. interrogans	Australis	Australis	Ballico
L. interrogans	Australis	Bratislava	Jez Bratislava
L. interrogans	Autumnalis	Rachmati	Rachmat
L. borgpetersenii	Ballum	Ballum	Mus 127
L. interrogans	Bataviae	Bataviae	Swart
L. interrogans	Canicola	Canicola	Hond Utrecht IV
L. weilii	Celledoni	Celledoni	Celledoni
L. kirschneri	Cynopteri	Cynopteri	3522 C
L. kirschneri	Grippotyphosa	Grippotyphosa	Mandemakers
L. kirschneri	Grippotyphosa	Grippotyphosa type Moska	Moskva V
L. interrogans	Hebdomadis	Hebdomadis	Hebdomadis
L. interrogans	Icterohaemorrhagiae	Copenhageni	Wijnberg
L. interrogans	Icterohaemorrhagiae	Icterohaemorrhagiae	Kantorowic
L. borgpetersenii	Javanica	Poi	Poi
L. borgpetersenii	Mini	Mini	Sari
L. noguchii	Panama	Panama	CZ 214
L. interrogans	Pomona	Pomona	Pomona
L. noguchii	Pomona	Proechimys	1161 U
L. interrogans	Pyrogenes	Pyrogenes	Salinem
L. borgpetersenii	Sejroe	Hardjo type bovis	Sponselee
L. interrogans	Sejroe	Hardjo type prajitno	Hardjoprajitno
L. borgpetersenii	Sejroe	Saxkoebing	Mus 24
L. borgpetersenii	Sejroe	Sejroe	M 84
*L. biflexa**	Semaranga	Patoc	Patoc I
*L. meyeri**	Semaranga	Semaranga	Veldrat Semarang 173
L. santarosai	Shermani	Shermani	1342 K
L. borgpetersenii	Tarassovi	Tarassovi	Perepelitsin

*Nonpathogenic leptospires. All others are pathogenic.

DNA Isolation

Total nucleic acid extraction from blood and urine samples was performed using The NucliSENS EasyMAG® automated system (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. Blood samples (K3-EDTA 1 ml tube), were suspended in 2 ml EasyMAG lysis buffer. Urine samples were centrifuged at 14,000× g 30 minutes at room temperature, the supernatant was discarded and the pellet was re-suspended in 2 ml EasyMAG lysis buffer. The DNA was eluted in 80 µl elution buffer in the last step of the extraction procedure.

TaqMan Real-time PCR

DNA extracted from cats' biological materials were tested with TaqMan real-time PCR described by Ahmed et al., 2020[25]. Briefly, primers and probe sequences targeting lipL32 gene-specific for pathogenic *Leptospira* (LipgrF2, LipgrR2, and LipgrP1) and the internal set primers, probe, and synthetic internal control template sequences (IntoF2, IntoR2, IntoP1, and PlasintS1) were listed in Table 2. The PCR analytical sensitivity for the spiked serum, blood, and urine with *L. interrogans* were estimated as 2, 3, and 5 leptospires per reaction respectively. The PCR as described has a high specificity and is capable of detecting all pathogenic *Leptospira* so far known. Between 100–500 copies per reaction of genomic DNA extracted from *Leptospira interrogans* strain Kantorowic was used as a positive control. The PCR was performed, including the internal control template to monitor the reaction performance and double-distilled DNase/RNase-Free water as a negative control. All clinical samples were tested in duplicate.

Table 2. Description of the sequence of *lipL32,* internal set primers, probe, and synthetic internal control used in the study.

Oligo ID	Sequence	Sequence source
LipgrF2	5'CGCTGAAATGGGAGTTCGTATGATTTCC3'	*lipL32*
LipgrR2	5'GGCATTGATTTTTCTTCYGGGGTWGCC3'	*lipL32*
LipgrP1	5'FAM AGGCGAAATCGGKGARCCAGGCGAYGG3'BHQ1	*lipL32*
IntoF2	5'TAGAATCATTGAATCTATCACATCTCATG3'	Internal Control
IntoR2	5'TTGAACTAAATGTAGACTAAAGATGATCG'3	Internal Control
IntoP1	5'TxRd TTCACATTAACATTCAATAATCAATCATGAA3'BHQ2	Internal Control
PlasintS1	5'CTATAGAATCATTGAATCTATCACATCTCATGTACTTCACATTAACATTCAATAATCAATCATGAATTAATTCAATTTCTGATATGAATCGATCATCTTTAGTCTACATTTAGTTCAATATATC3'	Internal Control artificial template

The concentration of the reagents and the cyclic amplification protocol were the following; 12.5 ul of 2x master mix (applied biosystem), 0.4 µM of each leptospires forward and reveres primer (LipgrF2 and LipgrR2), 0.2 µM of the leptospires probe (LipgrP1), 0.16 µM of each internal control primers (IntoF2 and IntoR2), 0.08 µM of internal control probe (IntoP2), 0.25 µl double-distilled DNase/RNase-free water and 0.29 pg (equivalent to 50 copy) of internal control DNA template (PlasintS1). Finally, 10 µl of DNA extracted from cats' DNA in a total volume of 25 µl were submitted to an amplification procedure using the CFX96 real-time PCR detection system (Bio-Rad, Amsterdam, The Netherlands). The cyclic amplification protocol consists of the following steps; initial DNA denaturalization and DNA polymerase activation at 95 °C for 5 minutes, 45 cycles of two steps, 95 °C for 20 seconds as denaturalization and 60 °C for 30 seconds

as hybridization, and annealing-extension steps for the probes and each forward and reverse primers respectively.

Statistical Analysis

Apparent prevalence, 95% CI and CI of a proportion were calculated for antibodies against *Leptospira* serovars, leptospiral DNA in blood, leptospiral DNA urinary shedding, FeLV, and FIV infection with OpenEpi (Andrew G. Dean and Kevin M. Sullivan, Atlanta, GA, USA)[26]. Descriptive statistics were performed for the calculation of medians, mean, SD, and range. For the possible risk factors for *Leptospira* infection, the linearity assumption was first guaranteed with multiple logistic regression. Age, gender, sampling season, and co-infections with FeLV/FIV were analysed for binary logistic regression as possible risk factors associated with *Leptospira* (antibodies presence, amplified *Leptospira* DNA in blood and/or urine). A P value <0.05 was determined as statistically significant. Statistical analysis was performed using a commercial software program (IBM SPSS-Statistics version 22, IBM © Armonk, NY, USA).

Results

In total, 90/244 cats were from Barcelona and 154/244 were from Cáceres, all were domestic short-haired, 131 cats were male and 113 were female, with ages from 3 months to 16 years (mean 1.8 years and SD 2.30). In addition, 17/244 were FeLV positive (7%, 95% CI: 4.24–11%) and 7/244 were FIV positive (2.9%; 95% CI: 1.3–5.6%). None of the animals were positive for both diseases

(FeLV/FIV). One hundred and thirty-five cats were sampled in winter, 58 in spring, 15 in summer, and 36 in autumn.

Seroprevalence

Serum was obtained from all animals and 10/244 cats (4.1–95% CI: 2.1–7.18%), 9 from Cáceres and only one from Barcelona; 8 males and 2 females with ages ranged from 6 months to 6 years, were seropositive (antibody titres ranged from 1:20 to 1:320) for at least one serovar. Only two cats had antibody titres ≥ 1:320 against the serovars Bataviae and Proechimys (Table 3).

The most common serovars involved in the study were Cynopteri (5/10 of seropositive cats) followed by serovars Ballum, Bratislava, Grippotyphosa, and Proechimys. Antibodies titres for at least two serovars belonging to two different serogroups were detected in 2 cats. None of the cats with antibodies shed pathogenic *Leptospira* DNA in their urine. All animals were negative against saprophytic strains included in the panel.

DNA Detection in Blood PCR

Due to the low volume of the blood sample obtained during sampling in some animals, DNA isolation from blood samples was only possible in 89/244 cats. Only one sample (8-month-old female from Barcelona), was positive (1.12%; 95% CI: 0.05–5.41%). This cat had no antibodies against *Leptospira* detected by MAT (Table 4).

Table 3. Seropositive Microscopic agglutination test (MAT) results among 244 cats tested in Spain.

Gender	Age y.o.	Origin	Titre	Species	Serogroup	Serovar	Strain	FIV	FeLV
F	1	C	1:20	*L. borgpetersenii*	Ballum	Ballum	Mus 127	N	N
M	0.5	C	1:20 1:20	*L. interrogans* *L. kirschneri*	Australis Cynopteri	Bratislava Cynopteri	Jez Bratislava 3522 C	N	N
M	1	C	1:20	*L. kirschneri*	Cynopteri	Cynopteri	3522 C	N	N
M	2	C	1:20	*L. kirschneri*	Cynopteri	Cynopteri	3522 C	N	N
M	2	C	1:40	*L. kirschneri*	Cynopteri	Cynopteri	3522 C	N	N
M	2	C	1:320	*L. interrogans*	Bataviae	Bataviae	Swart	N	N
M	5	C	1:20	*L. borgpetersenii*	Ballum	Ballum	Mus 127	N	N
F	1	C	1:20 1:20 1:80 1:40 1:20 1:80 1:320	*L. interrogans* *L. kirschneri* *L. kirschneri* *L. kirschneri* *L. interrogans* *L. noguchii* *L. interrogans*	Australis Cynopteri Grippotyphosa Grippotyphosa Pomona Pomona Autumnalis	Bratislava Cynopteri Grippotyphosa Grippotyphosa-M Pomona Proechimys Rachmati	Jez Bratislava 3522 C Mandemakers Moskva V Pomona 1161 U Rachmat	N	N
M	2	C	1:20 1:20	*L. interrogans* *L. noguchii*	Pomona Pomoma	Pomona Proechimys	Pomona 1161 U	P	N
nM	7	B	1:20	*L. borgpetersenii*	Sejroe	Sejroe	M 84	N	N

F: Female; M: Male; nM: Neutered male; C: Cáceres, Extremadura; B: Barcelona, Catalonia; N: Negative; P: Positive, FeLV: feline leukaemia virus, FIV: feline immunodeficiency viru

Table 4. Results of DNA detection in blood and urinary shedding by polymerase chain reaction (PCR) in cats from Spain.

Gender	Age y.o.	Origin	Blood DNA amplification by PCR (*n* = 89)	Urine DNA amplification by PCR (*n* = 232)	FIV	FeLV
F	1	C	N	N	N	N
M	0.5	C	N	N	N	N
M	1	C	N	N	N	N
M	2	C	N	N	N	N
M	2	C	N	N	N	N
M	2	C	N	N	N	N
M	5	C	N	N	N	N
F	1	C	N	N	N	N
M	2	C	N	N	P	N
nM	7	B	N	N	N	N
M	0.5	C	N	P	N	N
F	1	C	N	P	N	N
M	0.5	B	N	P	N	N
F	0.5	B	N	P	N	N
F	0.6	B	P	N	N	N

F: Female, M: Male, nM: Neutered male, y.o.: Years old, C: Caceres, B: Barcelona, U PCR: Urinary PCR, B PCR: Blood PCR, N: Negative, P: Positive.

Urinary Shedding

It was not possible to collect urine in 12 cats and therefore, 232 urine samples were processed for DNA extraction and subsequently PCR testing. A total of 4/232 samples amplified DNA from pathogenic *Leptospira* species (1.72%; 95% CI: 0.55–4.10%); two cats (1 male and 1 female) were from Cáceres and two cats (1 male and 1 female) were from Barcelona. All positive cats were ≥1-year-old (Table 4). All PCR negative controls tested negative and all PCR positive controls tested positive. None of these cats had antibodies against *Leptospira* by MAT.

Risk Factor Analysis

Multivariate logistic regression did not reveal significant risk factors for Leptospira infection neither for seroprevalence nor DNA detection in blood and/or urine in the present study; P values were ≥0.05.

Discussion

Cats are susceptible to *Leptospira*[9,10,12,27] and the presence of viable pathogenic leptospires in the urine of cats has been proven (p. 227,[19,28]). Therefore, the species can play a role in the transmission of the zoonosis. Cats are becoming more popular as a companion animal and it is therefore important to have data to assess the extent to which cats constitute a risk of human leptospiral infection.

Scant information is known about specific characteristics of leptospirosis pathogenesis in cats. Based on general knowledge of the disease, once an animal becomes infected it may develop the incidental host state with the presentation of acute illness, that may be fatal, or chronic renal carrier state with mild or non-presenting clinical signs[23]. There are some differences in the disease presentation linked to the infecting serovar[29]. Leptospires enter the warmer body environment and transcriptional changes occur that enhance their pathogenicity[23]. In rats and mice (chronic carrier host), the regulation of *Leptospira* lipopolysaccharide (LPS) differs from human and dogs (incidental host)[30,31]. In murine species, there is an adaptation of the innate response to infection with leptospires[32]. In carrier hosts (rats and mice), leptospires are disseminated through the organism and are most likely cleared by the immune

system from all tissues except the kidney. In the epithelial cells of the renal tubules, leptospires continue to multiply and are shed in the urine[33].

Based on experimental infections and previous reports on cats, leptospiraemia may be present in the first hours of infection[34], but on average, it appears from 6 days post-infection and lasts up to 7 more days[35]; antibody titre rises at the end of the first week of infection[34], the peak titre has been reported to be around day 21[35] but in many cases, cats unlike dogs, do not develop a high antibody titre[34–36]. In other species, antibodies last from months to years[2,23], but it has not yet been confirmed in cats. The shedding of leptospires in cats' urine appears from 2–4 weeks post-infection and it could last at a maximum of 6 weeks in case of acute disease (incidental host state)[34,35]. Acute cases of feline leptospirosis, however, are scarce nevertheless, epidemiological studies on leptospires prevalence demonstrate that the role of cats is mostly as a chronic carrier. In a cat, urinary shedding of pathogenic *Leptospira* has been demonstrated for a period of 8 months[15]. Based on the above information, it is our belief that cats as the murine species act most as the chronic carrier than an incidental host for the disease.

Seroprevalence against *Leptospira* observed in our study (4.1% positive 95% CI: 2.1–7.18%), fell within the previous intervals described worldwide, 4% to 33.3%[14,16,17,37]. Environmental factors such as outdoor habits, presence of farm animals that may shed leptospires in the neighbourhood, prey habits, or even the season of the year (resulting in different levels of exposure to pathogenic leptospires), can explain the broad ranges of antibody prevalence reported in the literature. Even different cut off values (≥1:100) and serovar panels used in laboratories may affect the prevalence. All these factors, along with a different

length of sampling (3 years) and sample size (*n* = 53), may explain the prevalence (14%) obtained previously in the country[38], compared with the lower prevalence in our study (4.1%).

Except for two cats from Cáceres (titre 1:320), antibody titres of the animals in our study were not ≥1:100. Adler, 2014[23], reported that infected animals may have MAT titres below 1:100; which is supported by epidemiological studies in cats[13,14,16,39]. Seropositive animals in our study demonstrate previous evidence of contact with *Leptospira*. They could have had a recent infection, as cats are not as routinely vaccinated against the disease as dogs, or they were chronically infected animals with falling antibody titres.

At the time of sampling, both cats with titres ≥1:320 showed no urinary shedding of *Leptospira* DNA. Nevertheless, we were unable to take further urine samples from these animals at different times (as per Weis et al., 2017)[15] in order to confirm if they had intermittent shedding of the bacteria DNA. To our knowledge, this is the first study in Spain to confirm by MAT, the seropositivity against serovars Bataviae, Bratislava, Cynopteri, Grippotyphosa, Pomona, Proechimys, and Rachmati in cats, suggesting the possible presence of serovars from serogroups Australis, Autumnalis, Bataviae, Cynopteri, Grippotyphosa, and Pomona among cats in the country. In the metropolitan area of Barcelona, Spain, the presence of members of serogroups Australis, Bataviae, and Grippotyphosa (also detected in our study in cats) has been detected in small mammals[40], so it may be possible for serovars to circulate between animal species. Another factor to consider is the increased contact between cats and animal reservoirs of leptospires, due to shifts in the dynamics and colonization of cities. Environmental changes often increase the frequency and magnitude of contact between wild

and domestic species, thus increasing the risk of disease transmission. Leptospirosis is an example of this dynamic interface with wildlife[41]. Serovars belonging serogroups Australis, Autumnalis, Batavie, Bratislava, Grippotyphosa, and Pomona have been previously described in cats from Europe[12,15,16,27,39,42].

A cross-sectional epidemiological *Leptospira* study was recently conducted in dogs from Spain[43]; however, serovars from serogroups Ballum, Bataviae, and Cynopteri, were not part of the diagnostic panel, unlike our study of cats which included them. Antibodies against serovars of these serogroups were present in the cats of our study. The variety and number of serovars and serogroups included in the diagnostic panel have a direct relationship with the sub-diagnosis of leptospirosis by MAT[44].

Generally speaking, cross-reactivity between leptospiral serogroups has been previously described. In dogs, antibody titres to heterologous strains may provide equal or higher titres than the infecting serovar[23]. A second test performed 15 days after the first one, could help determine the infecting serovar due to seroconversion. Although, still caution is needed since presumptive serogroup data should be used only to give a broad idea of the common serogroups present in a population and cannot be interpreted reliably in individual patients[45]. In the present study, 3 cats had antibody titres against more than one serovar (Table 3). Previously published studies[15,20,46] have also reported cats simultaneously seropositive against different serovars. The simultaneous seropositivity that some cats display, not only in our study but in others, could be explained by either a genuine cross-reactivity in cats or by the simultaneous exposure of the animals to different serovars belonging to different serogroups. In the case of the one-year-old female cat from Cáceres, she had antibodies against 7 different

serovars. Surely in the case of the serovars belonging to serogroup Pomona, it represents a cross-reaction with the highest titre obtained.

Cynopteri (belonging a Cynopteri serogroup) was the most frequently detected serovar, in our study. In all cases, the infected animals came from Cáceres; therefore, there is a possibility that a serovar from this serogroup is endemic in the cat population of this region. None of the seropositive cats shed *Leptospira* DNA in their urine. A plausible explanation for this could be that either the animals had an acute infection with increasing antibody titres at the moment of sampling and no shedding of *Leptospira* DNA had occurred yet, or most likely that they were chronic carriers with falling or steady antibody titres and non-continuous *Leptospira* shedding in the urine. Similar findings have been previously described[14,15,18,20].

According to the World Health Organization's guidance[24], the diagnostic panel of antigens used in MAT should include local strains, which increases the sensitivity of the technique compared to reference strains. However, the range of serovars should not be limited to local strains as the infection may be caused by a rare serovar or a strain not previously described. For this reason, we included saprophytic strains (*L. biflexa* strain Patoc I, *L. biflexa* strain CH 11, and *L. meyeri* strain Veldrat Semarang 173) in our panel diagnostic, which can cross-react with the antibodies generated by some pathogenic serovars. Possible explanations for the fact that none of the cats with antibodies against pathogenic leptospires had antibodies against the saprophytic serovars included in our MAT panel, could be that they have not either specific cross-reactivity against the used saprophytic ones or because as it has been described previously, the saprophytic serovars,

specifically serovar Patoc, has limited ability to detect cross-reactions with antibodies of past infections[47].

The presence of leptospires in cats' blood has been reported in clinical cases and in epidemiological research[6,10]. In our study, isolation of pathogenic *Leptospira* DNA in blood was only possible in 1 out of 89 cats. This cat was negative to FeLV/FIV tests, was seronegative against *Leptospira* and did not shed pathogenic leptospires DNA by urine. Therefore, we conclude that the animal was at an early stage of leptospirosis, with clinical signs non-present.

One epidemiological study in Taiwan, using serum and urine samples of cats[6], reported prevalence by PCR of 19.1% in blood and 67.8% in urine respectively. The differences in prevalence between this study and ours may be due to several factors such as i) Different primers used between studies. Chan et al., 2014[6] used two sets of primers, the first one *Leptospira* rrs (16S) which is not able to differentiate between non-pathogenic *Leptospira biflexa* and pathogenic *Leptospira spp.* The second one primer set G1/G2 amplified a 285-bp sequence by PCR from strains of all pathogenic *Leptospira spp*. except for *Leptospira kirschneri.* This fact leads to differences between sensitivity and specificity in the PCR techniques used between studies. ii) The origin of the cats sampled in Taiwan; most of them came from rural areas; iii) The climatic conditions related to Taiwan, where typhoons are frequent and therefore favour the conditions for the maintenance of the leptospires in the environment; and iv) They used a random sample of cats which included shelter and household cats**.**

The prevalence of urinary shedding from leptospiral DNA in the present research is consistent with that reported in previous studies in cats[6,7,14,15,18,19]. Our results

also match with those obtained by Sprißler et al., 2018 (0.8%)[14] and Gomard et al., 2019 (0.6%)[8]. In common, the two PCRs methodologies used in these two studies and in our work are targeting the *lipL32* gene[25,48]. In a sample of healthy cats and cats suffering from kidney disease, Rodriguez et al., 2014[20] described a urinary prevalence of 14.9% in the latter group. Our results are not comparable with those, as the animals in our study were part of a free-roaming or shelter neutering program and at the time of sampling, none of the 4 cats showed clinical signs of disease. In cats, the urinary shedding time of *Leptospira* DNA remains unknown. Urine cultures of the cats in our study were not carried out due to the cumbersome and time-consuming nature of the methodology. The fact that none of the urinary shedding cats had antibody titres by MAT, may indicate that they have been chronically infected animals with falling, steady, or non-present antibody titres at the moment of sampling[35,36]. The MAT test has limitations detecting renal carriers[23]. The panel used in our study was broad, 8 species of *Leptospira*, belonging to 20 serogroups, 27 serovars, and 28 strains even including non-pathogenic serovars, so we can rule out the possibility that the *Leptospira* infection was sub-diagnosed.

One limitation of the research was that it was not possible to perform a follow up of tests of the cats, in order to determine seroconversion and the urinary shedding of pathogenic *Leptospira* DNA over time. This was due to the cats' origin, as mentioned above.

Conclusions

In conclusion, this study reports seropositivity by MAT, against serovars belonging serogroups Australis, Autumnalis, Bataviae, Cynopteri, Grippotyphosa, and Pomona, for the first time in cats from Spain. Moreover, the antibodies against serovar Cynopteri (serogroup Cynopteri) were the most frequently detected in cats from Cáceres (Extremadura, southwest Spain). Knowledge of the involved leptospiral serovars in animals from any country is imperative for the accurate diagnostics and epidemiological surveillance of the disease. Therefore, we recommend including at least the aforementioned serogroups in any MAT panels used by diagnostic laboratories for detecting leptospiral antibodies in cats as well as dogs from Spain.

To our knowledge, this is the first report of urinary shedding of pathogenic leptospiral DNA in cats from Spain, diagnosed by means of molecular tools targeting the *lipL32* gene. Free-roaming cats in Spain can shed *Leptospira* DNA in their urine and may be a source of infection for people. Although the presence of viable leptospires in urine culture from cats has already been shown (p. 227,[19]), more prospective studies should be performed to ascertain the role of cats in the spread of the zoonosis.

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4.2. STUDY II

Clinicopathological findings in cats naturally infected by pathogenic *Leptospira spp.*

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Abstract

Background –Cats infected with *Leptospira spp.* are an incidental host or chronic carrier of the diseases. Clinical signs are mild or non-present. There is a lack of information about the clinicopathologic findings in naturally infected cats by pathogenic leptospires.

Objective -The present study aimed to determine differences in haematology, biochemical profile and urine parameters between naturally infected by *Leptospira spp.* and leptospires-free cats.

Animals – Urine, blood and serum from free-roaming cats from Catalonia and Extremadura, Spain, tested against pathogenic leptospires by microscopic agglutination test (MAT) and polymerase chain reaction (PCR).

Methods – Case-control study. Haematology, biochemical profile and urinalysis were performed in 15 cats naturally infected by leptospires, confirmed by MAT and PCR tests and in 19 leptospires- free cats.

Results – Cats naturally infected by pathogenic leptospires had lower values RBC (*P-value* 0.00), Hb (*P-value* 0.00), albumin (*P-value* 0.01), creatinine (*P-value* 0.01*)* and Urea (*P-value* 0.01*)* compared to leptospires-free cats. DNA amplification cats were at high risk for the development of anaemias (low RBC and Hb) when compared with leptospires- free cats (*P*-value 0.03). In contrast, Seropositive cats were more likely to have proteinuria when compared with leptospires-free cats (*P*-value 0.03).

Conclusions and clinical importance – Chronic, infection and exposure, to leptospires leads to haematological abnormalities and slight alterations in the biochemical profile and urinalysis.

Key Words: Anaemia, Microscopic Agglutination Test, Polymerase Chain Reaction, Proteinuria, Seropositive.

Introduction

Leptospirosis is one of the most widespread and neglected zoonotic diseases worldwide[1]**.** It has also become an emerging disease driven by climate and environment changes e.g., temperature, humidity, rainfall, and a rise in sea-level, disturbs transmission dynamics of the disease[2]. Changes in ecosystem dynamics such as the close contact between domestic and reservoirs animals of *Leptospira spp.* increase the risk of disease transmission[3]. Leptospires are spread into the environment by the urine of infected animals, mainly mammals[4] including domestic cats[5], infecting soil, surface water, streams and rivers.

Antibodies presence against the bacteria[6-8], *Leptospira spp.* DNA urinary shedding[9-12] and even viable pathogenic leptospires urinary shedding[13-14]; have been described in cats from several continents.

Once a cat becomes infected it may develop an acute illness (incidental host state) with clinical signs, usually mild (rarely severe or fatal); therefore, it can be underdiagnosed[15-17]. Humans infection is an example of an incidental host[18].

Alternatively, cats would develop instead a chronic renal carrier state with no clinical signs[11,14,19] depending on several factors, including the infecting serovar,

bacterial load, host immunological competence and age, among others[18,20]. Some animal species are the chronic renal carrier for some *Leptospira* serovars e.g., rats for Icterohaemorrhagiae and Copenhageni[21], dogs are for Canicola, cows and sheep for Hardjo and pigs for Pomona and Bratislava[18]. It is known that the renal carrier state is a key component which is central to the persistence and epidemiology of leptospirosis[22]. The renal carrier state in cats has been documented on several continents, with a prevalence ranging from 0-67.8%[7,12,14,23].

Since clinical symptoms depend on whether the animal has an acute process, with mild symptoms, or develops a chronic carrier state, the diagnosis of leptospirosis can be challenging. Information on clinical leptospirosis in domestic cats is scarce, and features in clinicopathologic parameters in affected animals can add valuable information for future research in the disease which remains poorly studied compared to dogs[24,25].

To our knowledge, only a handful of studies reported haematology values, biochemistry screen or urinalysis data associated with leptospirosis in cats, most of them from acute[15-17,26] or experimental infections[27-29]. Both leukocytosis or leukopenia have been reported in acute leptospirosis in cats[26,30]. Renal azotemia[6,11,31], proteinuria, haematuria, and isosthenuria or low ability to concentrate urine[15,17] are other described modifications in cats. No changes in haematology and low urinary specific gravity (USG), have been reported, however, in acute experimental infections[27-29].

The present study is therefore aimed to determine differences in haematology, biochemical profile and urinary parameters between naturally infected cats by *Leptospira spp.* and leptospires-free cats.

Materials and methods

Animals

The study was designed as a case-control study. Blood and urine samples were obtained from cats that were part of a free-roaming spay program (Extremadura, Spain) and a neutering shelter program (Catalonia, Spain) between October 2017 and September 2018. Cats were grouped initially as naturally infected by pathogenic leptospires (n=15) and leptospires- free cats (n=19). *Leptospira spp.* infected cats were further divided in i) seropositive cats to at least one serovar of *Leptospira spp.* (Group1, *n* = 10) and ii) cats with pathogenic leptospires DNA amplification by PCR in blood or urine (Group 2, *n* = 5). Leptospires-free cats were seronegative for antibodies against *Leptospira* spp. and did not amplify for leptospires' DNA (Group 3, *n* = 19).

Serum antibodies presence against *Leptospira spp.* (evaluated via Microagglutination test-MAT, the panel test included 27 serovars, belonging to 21 serogroups and 8 species of *Leptospira*) and blood and urine pathogenic leptospires DNA amplification by PCR was also performed in the animals as part of a previous epidemiologic study.

All animals were tested for the presence of feline immunodeficiency virus (FIV) antibody and feline leukaemia virus (FeLV) antigen using a commercial enzyme-

linked immunosorbent assay (SNAP Combo FeLV/ FIV test IDEXX®). The study was approved by the Commission of the Doctorate Program in Medicine and Animal Health of the Autonomous University of Barcelona (UAB), approval CEEAH, code 2939.

Samples

Blood samples (3mL) were collected via jugular venipuncture with the animals under general anaesthesia. One millilitre of the sample was transferred in a K3-EDTA tube and 2 mL to a tube containing a coagulation activator. After clot formation, the tubes were centrifuged at 1,300 X *g* for 10 minutes. The serum obtained was stored at -20°C until biochemistry testing. Urine was collected in sterile syringes and refrigerated. Haematology and urine analysis were performed within an hour of collection.

Variables of the study

The following haematological variables were studied: red blood cell count (RBC), haemoglobin concentration (Hb), haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), white blood cell count (WBC) and differential leukocyte count and platelet count (PLT). All analytes were determined using an automated laser cell counter (ADVIA 120, Siemens Healthcare Diagnostics, Deerfield, IL, USA) with multispecies software that allows automatic differential leukocyte counts to be performed of several species. Internal controls are run daily to ensure that the system is in analytical calibration (ADVIA 120 3 in 1 Test Point, Siemens Healthcare Diagnostics, Deerfield, IL, USA). Serum concentrations of albumin, globulins, A/G ratio, total protein (TP), creatinine, urea, and alanine aminotransferase (ALT), were determined using an automated analyser (Olympus AU600, Olympus Diagnostic, GmbH, Tokyo),

Urinalysis (urinary specific gravity (USG) determined by refractometry and urine dipstick multiple chemical test strips, Dialab® GmbH, Austria) including microscopic examination of the urine sediment were determined.

Statistical analysis

Median, standard deviation, and range were calculated for all parameters. Normal distribution of data was assessed by kurtosis, skewness, and the Shapiro-Wilk test. As the data were not normally distributed, a non-parametric analysis was done. A comparison of infected by leptospires versus leptospires-free cats was performed using Mann-Whitney U-test. The Kruskal-Wallis one-way analysis of variance in ranges was performed to compare the mean between groups. Differences in proportions of abnormal results (proportions above or below of reference intervals) between infected by leptospires and leptospires-free cats for CBC, biochemistry and urine parameters were evaluated by constructing 2x2 contingency tables, with subsequent analysis using a Fisher's exact test. The odds ratio (OR), 95% confidence interval and *P*-value were calculated for 34 parameters. The parameters in which their increase or decrease have no clinical relevance were not included in the Fisher's exact test. A *P*-values < 0.05 were considered statistically significant. Statistical analysis was performed using R Statistical software 3.6.2 version[32], libraries “gplots” (3-0-1-2 version)[33] and “pgirmess” (1-6-9 version)[34].

Results

34 short-haired cats were included in the study (12 females and 22 males, ranging from 6 months to 12.5 years old). The group of seropositive to *Leptospira*

spp. (group 1), were 10 cats (2 females and 8 males, from 6 months to 7 years old). One cat of this group had co-infection with FIV. The group of DNA amplification by PCR (group 2), were 5 cats (3 females and 2 males, from 6 months to 1 year old). All cats were negative for FeLV/FIV test in this group. The group of leptospires-free cats (Group 3), were 19 cats (7 females and 12 males from 6 months to 12.5 years old). All cats were negative for FeLV/FIV test in this group. Cats in the infected and leptospires-free groups did not differ regarding age (*P*-value 0.24) or gender (*P*-value 0.31). There was, however, a statistically significant difference in the age distribution between the three groups comparison (*P*-value 0.02); animals from group 2 were younger than those of groups 1 and 3.

Table 1 shows the median, minimum and maximum values of haematology, biochemistry and urinary analysis results, reference intervals, and comparison of median values between naturally infected to pathogenic leptospires (seropositive and positive DNA amplification by PCR) and leptospires-free cats. Odds ratio (OR) and 95% confidence interval (CI) of the proportion of naturally infected to pathogenic leptospires or leptospires-free cats, with laboratory results above (high) or below (low) the reference interval are presented in Table 2.

Infected cats have significantly lower values of RBC (*P-value* 0.00), Hb (*P-value* 0.00) albumin (*P-value* 0.01), creatinine (*P-value* 0.01) and urea (*P-value* 0.01) than leptospires-free cats (Table 1). When subdividing infected cats, positive DNA amplification by PCR group, have significantly lower Hb concentration (*P-value* 0.00) and haematocrit percentage (*P-value* 0.01) than the leptospires-free group. The seropositive group had significantly lower urea (P-*value* 0.00) values compared to the leptospires-free cats (Table 1).

Table 1. Median, minimum and maximum values of haematology, biochemistry and urine analysis, reference intervals, and comparison of median values between naturally infected to pathogenic leptospires cats (seropositive and positive DNA amplification by PCR) and leptospires-free cats.

	INFECTED						**LEPTOSPIRES-FREE**		
	Total Infected *n* = 15		**Seropositive *n* = 10**		**Positive DNA amplification by PCR *n* = 5**		**Control *n* = *19***		**Reference Interval**
Parameter	Median	Min-Max	Median	Min-Max	Median	Min-Max	Median	Min-Max	(RI)
RBC (10^{12}/L)	7.24**	4.47-8.75	7.47	6.16-8.75	5.97	4.47-8.38	8.53	6.41-11.46	6-10.2
Hb (g/dL)	11.25**	7.20-14.20	11.90	10.20-14.20	9.10**	7.20-11	13.30	11-17.50	9-15
Haematocrit (%)	32.05	19.80-41.50	34.50	28.20-41.50	26.35**	19.80-30.30	34.80	28.60-47.20	29-48
MCV (fl)	47.15	35.60-55	47.40*	37.80-55	43.10	35.60-48.60	40.90	31.80-53.20	41-53
MCHC (g/dl)	34.70	32-38.70	35.55	32.10-38.70	33.75	32-36	35.90	32-38.60	30-34
WBC (10^9/L)	12.25	6.72-23.39	12.32	6.86-20.72	11.09	6.72-23-39	9.99	4.20-18.37	5-15
Lymphos (10^9/L)	3.41	1.61-5.84	2.99	1-61-5.84	4.02	2.47-5.14	2.56	0.84-6.62	1.4-6.1
Monos (10^9/L)	0.33	0.02-1.10	0.30	0.02-1.10	0.34	0.09-0.46	0.26	0.07-0.85	0.1-0.6
Segs (10^9/L)	7.06	3.78-17.20	7.50	4-16	6.06	3.78-17.20	5	2-13.25	2.5-11.3
Eos (10^9/L)	0.53	0-1.39	0.65	0.18-1.15	0.28	0-1.39	0.71	0-1.18	0-1.5
Basos (10^9/L)	0.01	0-0.09	0	0-0.09	0.01	0-0.02	0	0-0.07	0-0.1
PLT (10^9/L)	131.50	24-381	131.50	24-381	134.5	27-302	219	27-423	200-600
Albumin (g/L)	28.20*	21.20-32.90	28.55	23.10-32.90	26.3	21.2-32.2	31.30	24.80-44.90	23-34
Globulins (g/L)	44	28.60-68.70	43.60	31.70-68-70	44	28.60-52.50	42.80	24.80-52.40	26-38
A/G Ratio	0.63	0.40-1.05	0.67	0.41-0.95	0.56	0.40-1.05	0.8	0.52-1.04	>0.8
TP (g/L)	69.40	58-96.90	70.10	58-96.90	68.8	58.60-73.70	74	50.60-95.60	54-78
Creatinine (µmol/L)	91.50*	50.39-106.08	91.06	50.39-106.08	91.50	58.35-102.55	100.78	64.53-161.78	44.2-132.6
Urea (mmol/L)	7.44*	4.16-9.89	6.98*	4.16-8.66	8.57	5.48-9.89	8.37	5.94-11.41	3.33-8.33
ALT **(**U/L)	27.90	7.50-51.40	29.20	7.50-44.70	27.90	24.70-51.40	35.40	5.94-11.41	< 50
URINE ANALYSIS^									
USG^^	1.050	1.030-1.050	1.050	1.030-1.050	1.050	1.046-1.050	1.050	1.030-1.050	> 1.035
pH	6.5	6-8	6.75	6-8	6	6-7	6.5	6-8	6-7.5
Protein (mg/dL)	100	0-300	200	0-300	100	0-100	100	0-300	0-100
Glucose (mg/dL)	0	0	0	0	0	0	0	0	0

Min = Minimum, Max = Maximum, ^Urine parameters were determined by dipstick, ^^Determined by refractometer, *Significant difference $P < 0.05$, **Significant difference $P < 0.01$.

Table 2. Odds ratio (OR) and 95% confidence interval (CI) of the proportion of naturally infected to pathogenic leptospires or leptospires-free cats, with laboratory results above (high) or below (low) the reference interval.

			Infected versus leptospires-free			**Seropositive versus leptospires-free**			**Positive DNA amplification by PCR versus leptospires-free**		
Parameter	Leptospires-free (%)		Infected (%)	*P*-value	OR (CI)	Seropositive (%)	*P*-value	OR (CI)	PCR (%)	*P*-value	OR (CI)
Origin	Cat	47	27	0.3	0.4 (0-2.1)	10	0.09	7.6 (1-77.1)	60	1	0.6 (0-7)
	Ext	53	73			90			40		
Gender	Female	37	33	1	0.9 (0.1-4.4)	20	0.43	2.3 (0.3-28)	60	0.6	0.4 (0-4.5)
	Male	63	67			80			40		
Neutering	Yes	21	7	0.4	3.6 (0.3-196.5)	10	0.42	0.6 (0-5.3)	0	0.5	0 (0-6.2)
	No	79	93			90			100		
Age	Young	37	60	0.3	0.4 (0-1.9)	40	1	1.1 (0.1-7)	100	0.03*	N/A (1.1-N/A)
	Adult	63	40			60			0		
RBC (10^{12}/L)	Low	0	20	0.08	0 (0-1.8)	10	0.34	N/A	40	0.03*	N/A (1- N/A)
	High	16	0	0.24	N/A	0	0.53	0 (0-4.6)	0	1	0(0-10)
HB (g/dL)	Low	0	13	0.19	0 (0-4.1)	0	N/A	N/A	40	0.03*	N/A (1- N/A)
	High	21	0	0.11	N/A	0	0.28	0 (0-2.8)	0	0.5	0 (0-6.3)
Haematocrit (%)	Low	5	27	0.15	0.2 (0-1.9)	20	0.27	4.2(0.2-279.2)	40	0.1	10.2 (0.4-740.5)
	High	5	0	1	N/A	0	1	0 (0-74)	0	1	0 (0-148)
WBC (10^9/L)	Low	10	0	0.5	N/A	0	0	0 (0-10.2)	0	1	0 (0-22)
	High	10	33	0.2	0.2 (0-1.9)	40	0.14	5.3 (0.59-73)	20	0.5	2 (0-50)
Lymphos (10^9/L)	Low	10	0	0.5	N/A	0	0.5	0 (0-10.2)	0	1	0 (0-22)
	High	5	0	1	N/A	0	1	0 (0-74)	0	1	0 (0-148)
Monos (10^9/L)	Low	12	33	0.2	0.2 (0-1.9)	40	0.14	5.3 (0.6-73)	1	0.5	2 (0-50)
	High	10	13	1	0.8 (0-12)	20	0.6	2 (0.1-33.4)	0	1	0 (0-22)
Segs (10^9/L)	Low	10	0	0.5	N/A	0	0.53	0 (0-10.2)	0	1	0 (0-22)
	High	10	33	0.2	0.2 (0-1.9)	40	0.14	5.3 (0.59-73)	20	0.5	2 (0-50)
Basos (10^9/L)	High	16	13	1	1.2 (0.1-16.6)	10	1	0.6 (0-8.9)	20	1	1.3 (0-23)
PLT (10^9/L)	Low	42	67	0.19	0.4 (0-1.8)	70	0.2	3.1 (0.5-24.4)	60	0.6	0 (0.2-29.3)
Albumin (g/L)	Low	0	13	0.19	0 (0-4.1)	10	0.3	N/A	20	0.2	N/A
	High	21	0	0.11	N/A	0	0.3	N/A	0	0.5	0 (0-6.2)
	Low	5	0	1	N/A	0	0.3	0 (0-2.8)	0	1	0 (0-148)

Globulins (g/L)	High	58	73	0.5	0.5 (0-2.6)	80	0.4	2.8 (0.4-34.2)	60	1	1.1 (0.1-16)
A/G Ratio	Low	47	73	0.17	0.3 (0-1.7)	80	0.1	4.2 (0.6-51.1)	60	1	1.6 (0.1-24)
TP (g/L)	Low	5	0	1	N/A	0	1	N/A	0	1	0 (0-148)
	High	12	13	0.26	2.9 (0.4-34.7)	20	0.7	0.6 (0-4.2)	0	0.3	0 (0-3.3)
Creatinine (µmol/L)	High	26	0	0.05	N/A	0	0.1	0 (0-1.9)	0	0.5	0 (0-4.4)
Urea (mmol/L)	High	58	33	0.18	2.6 (0.5-14.3)	10	0.02	0.09 (0-0.9)	80	0.6	2.8 (0.2-161)
ALT **(**U/L)	High	5	0	1	N/A	0	1	0 (0-74)	0	1	0 (0-148)
URINE ANALYSIS											
USG	Low	11	7	1	1.6 (0-104)	10	1	1 (0-20)	0	1	0 (0-22)
pH	High	21	13	0.67	1.7 (0.2-21.8)	20	1	0.9 (0-1.4)	0	0.5	0 (0-6.2)
Protein (mg/dL)	High	10	33	0.2	0.2 (0-1.9)	50	0.03*	7.8 (1-106)	0	1	0 (0-22)

* Significant difference $P < 0.05$, N/A: not applicable, Cat: Catalonia, Ext: Extremadura

Leptospira seropositive cats' group were more likely to have proteinuria when compared with leptospires-free cats (Table 2). In contrast, positive DNA amplification by PCR group cats, was at high risk for the development of anaemias (low RBC and Hb) when compared with leptospires-free cats (Table 2).

Discussion

Age was statistically significantly lower (*P*-value 0.02) in animals from group 2 compared with those of the groups 1 and 3. This agrees with Millan[35]; et al in cats and Craig, E.; et al[36], in dogs who observed that leptospirosis is more frequent in young animals. Anaemia, hypoalbuminemia, and low creatinine and urea values were the most relevant clinicopathological findings in cats naturally infected by pathogenic leptospires. Anaemia was the most likely disorder in cats that amplified DNA by PCR (urine or blood), unlike seropositive cats with antibody titres (linked to a chronic presentation), which could develop proteinuria.

Few studies have been conducted on the clinicopathological data of leptospires infection in cats, and most reports of them are from animals in acute disease state while others are from experimental infections. The changes in different haematological and serum biochemical variables and a few parameters of the urinalysis obtained from cats naturally infected by *Leptospira spp*. and the comparison with those obtained from leptospires-free cats are discussed below.

The lower RBC and Hb in the infected cats are indicative of mild anaemia, normocytic, and normochromic based on erythrocyte indices (non-regenerative with residual normal erythrocytes), as previously described in chronic

inflammation caused by infectious and non-infectious diseases[37-39]. According to MAT and PCR test results of our study, 14/15 cats had a chronic infection by *Leptospira spp.* rather than acute infection (incidental host). In addition to leptospirosis, due to the free-roaming nature of the animals of our study other than FIV/FeLV infectious diseases, cannot rule out as contributors to the inflammation. Such anaemia may also have a nutritional origin due to combined protein, vitamin, and mineral deficiencies[40].

The mild anaemia described here is consistent with the results published in a review on feline leptospirosis[26], which collected data from leptospires infection and reflected an incidence of anaemia of 10.5% in chronic carrier cats. In contrast, experimental studies of acute leptospirosis in cats did not describe anemia[27-29].

In canine leptospirosis, the presence of anaemia associated with different pathogenic mechanisms has been described (e.g. acute oliguric renal failure with overhydration; decreased erythrocyte production from chronic renal failure; blood loss from tissue thrombosis and/or acute pulmonary or gastrointestinal tract haemorrhage)[24-25,36,41]. Based on the unremarkable physical exam and the clinicopathological data of the animals in our study, those conditions as causes of anaemia can be ruled out. Anaemia secondary to haemolysis, due to the effects of leptospiral toxins, has been described mainly in cows and to a lesser extent in dogs[42]; in cats, however, it has not been described so far. The fact that cats which amplified pathogenic leptospires species DNA by PCR in our study (mainly chronic renal carriers of the bacteria), had a greater risk for developing anaemia, supports the hypothesis that a chronic condition (possible due to *Leptospira spp*. infection) is the most likely cause of it.

According to previous reports in dogs[41,43] and humans[44-45], leukocytes and platelets are the CBC parameters more often disturbed in leptospirosis. Nevertheless, there were no statistically significant differences between groups in our study in these parameters. Leukocytes comprise essential components of the innate immune response in the host[18], but their role in protection against leptospires infection in cats is unclear. Resistance to phagocytosis has been suggested as one of the leptospires potential virulence factors[46]. It is likely that other unclear factors of virulence, play a role in the suppression of interleukins production and therefore in the leukocytes and platelets synthesis. It should be noted that in *Leptospira spp*. infection, the carrier host has a long-term evolutionary association with leptospires in which a balance is reached between virulence and host response, making the organism almost commensal[18].

Infected animals of our study presented lower values of albumin, although within reference values, associated with normal-proteinemia than those of leptospires-free group. Decreased albumin can be attributed to either albumin loss (e.g., renal disease, gastrointestinal disease, intestinal parasitism) or failure of albumin synthesis. In some forms of gastrointestinal disease, protein and nitrogen losses occur so that the loss of albumin impairs its synthesis at the liver[39]. However, no gastrointestinal tract signs were observed in these animals either during the physical exam or after the spay. Intestinal parasitism cannot be ruled out in our animals, as diagnostic tests for faecal flotation were not performed, and their deworming history was unknown, but we believe that in that case, serum albumin values would have been lower than the reference values. A renal disease that enables albumin glomerular filtration has also to be ruled out due to urea, and creatinine serum concentrations were not increased.

Serum albumin is a negative acute-phase protein, and the concentration of this protein falls gradually with extensive inflammation, with the reduction in concentration being more noticeable in chronic inflammatory disease[47]. This fall accompanied of the vasculitis associated with the inflammatory process triggered by *Leptospira* lipopolysaccharides (LPS), which increases the vascular permeability leading albumin leakage to the interstitial space[36] may compound the lower values of albumin observed in the infected cats.

A dietary protein deficiency is another factor that, in addition to the above, could explain the decreased albumin concentration in our infected animals. Because of the sensitivity of albumin synthesis to protein and nitrogen availability, decreased albumin concentration precedes the development of generalized hypoproteinaemia in this situation[39].

The observed lower creatinine values in infected cats could be associated with reduced muscle mass in these groups, comprised of younger animals than those in the leptospires-free group. Creatinine increases with body weight[48] and age[49]. The infected cats also had lower urea serum values. Plasma or serum urea concentrations much depends on protein supply[50-51] and fasting concentrations of plasma urea are lower in cats on low-protein diets with normal or reduced renal function[52]. The nature of the cats in this study makes us consider low dietary protein intake as the most likely cause of this finding.

The group of cats with antibodies had a greater risk of developing proteinuria. However, the fact that in none of the animals, the values exceeded 200mg/dl, they did not have active sediment, and the USG was in normal values, allows us to say that this proteinuria was mainly of tubular and not a glomerular origin.

Leptospires are localized in the proximal renal tubules where they multiply and are voided in the urine for a variable period[18]. In dogs with acute leptospirosis, proteinuria is reported in 60% or more of cases[41,53-55].

It is very likely that in cats, due to long-lasting exposure to leptospires, a tubular dysfunction will be triggered that chronically affects the absorption of low and medium molecular weight proteins, while urea and creatine values, as of late indicators of kidney damage, remain within the reference values. Nevertheless, this is merely a hypothesis, and further specific tests (e.g., urinary electrophoresis or kidney biopsies) should be conducted to elucidate the mechanisms and significance of our finding and whether proteinuria is glomerular or tubular in origin[54,56]. A previous study conducted on leptospirosis in wild and domestic carnivores[35] reported histopathologic lesions (chiefly chronic interstitial nephritis or chronic inflammatory infiltrated) in feral cats with evidence of contact with serovars of the species *L. interrogans*. Some potential limitations of this study are the small sample size, the failure to determine the presence of seroconversion through a second MAT test, and the lack of clinical history of the animals. Had this been the case, our results could have been more conclusive.

Conclusions

Here we report the haematology values, biochemistry screen, and urinalysis parameters from cats naturally infected by *Leptospira spp***.** Cats naturally infected by pathogenic leptospires can develop non-regenerative anaemia. Those that amplify pathogenic *Leptospira spp.* DNA by PCR (urine or blood), are at higher

risk of developing it while *Leptospira spp.* seropositive cats, with antibody titres linked to a chronic presentation, are at higher risk of developing proteinuria.

Clinicopathological data associated with leptospirosis in cats are scarce, and most of them are from acute or experimental infections. As underdiagnosis of the disease in this species (clinical signs are usually mild) could lead to a substantial public health risk, the results published in our study would provide valuable insights for clinicians in diagnosing chronic renal carrier state of *Leptospira spp*. in cats.

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4.3. STUDY III

Acute phase proteins and total antioxidant capacity in free-roaming cats infected by pathogenic leptospires

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ABSTRACT

Background: Leptospirosis is a neglected but widespread zoonotic disease throughout the world. The vast majority of mammals are hosts of *Leptospira spp.,* including domestic cats. Though clinical signs of active leptospirosis are generally mild in cats, chronic infections might result in renal injury. However, there is no consensus either on the clinical presentation nor on the disease diagnosis in cats. The study of acute-phase proteins (APPs) and biomarkers of oxidative status would contribute to knowledge about the development of

the disease in cats. In this report, we evaluated four APPs: Serum amyloid A-SAA, Haptoglobin–Hp, albumin and Paraoxonase 1-PON1 and the antioxidant response through Total Antioxidant Capacity-TAC, in 13 free-roaming cats naturally infected by pathogenic leptospires and 19 leptospires-free cats, through a Principal Component Analysis (PCA).

Results: DNA positive cats showed higher serum SAA and Hp concentrations than seropositive cats and their leptospires-free counterparts. DNA positive individuals, however, had lower serum PON1 activity and albumin concentrations than seropositive and leptospires-free cats. On the other hand, the increase in TAC serum concentrations was positively associated with anti-leptospiral antibodies titre. The PCA retained two principal components (PC1 and PC2), explaining 60.1% of the observed variability of the inflammatory proteins and the antioxidant TAC. PC1 was mainly associated with an inflammatory process, whereas PC2 correlated with the antioxidant status. The most contributing variables in PC1 where albumin (27.46%), SAA (24.71%), Hp (21.56%) and PON1 (20.69%). Variables with significant contribution to the PC2 were the antibody titre against *Leptospira spp.* (48.41%) and TAC (35.04%). Overall the

PCA revealed differences in inflammatory and antioxidant biomarkers in cats naturally infected with leptospires compared to leptospires-free cats.

Conclusions: Increases in Serum SAA, Hp, and decreases in serum albumin concentrations and PON1 activity indicate an acute phase response in infected, DNA positive cats. Moreover, we found an increase in TAC serum concentrations indicating an antioxidant response in this infection, which is proportional to the antibody titre.

Keywords: Albumin, Cat, Haptoglobin (Hp), *Leptospira spp.,* Paraoxonase1 (PON1), Principal Component Analysis (PCA), Serum Amyloid (SAA) and Total Antioxidant Capacity (TAC).

Introduction

Leptospirosis caused by a spirochaetal bacterium of the genus *Leptospira* is a common and widely distributed zoonoses affecting livestock, domestic, and wild animals throughout the world[1]. A wide variety of mammalian species may be susceptible to *Leptospira spp.* infection including domestic cats[2] in America[3-5], Asia[6-7], Australia[8] and Europe[9-10].

Though leptospirosis in cats was suspected for years[11], it took two decades to be fully confirmed by PCR and serological methods[12-13]. Today, the role of cats as *Leptospira spp.* reservoir is well established[14-15] as is the acute disease caused by the infection[12,16-17].

Leptospires spread throughout the entire body, reproducing in different organs such as the kidneys, liver, central nervous system, eyes, and reproductive system. In cats, most of the *Leptospira spp.* infections are asymptomatic and unspecific and rarely results in severe organ damage[18]. Few works have described clinical signs related to the infection in cats (e.g., renal injury)[4,12,16,19], most of them being nonspecific[17,20-21]. As a result, there is no consensus on the clinical presentation, development and diagnosis of leptospirosis in domestic cats. Hence ancillary tests inflammatory proteins and biomarkers of oxidative status could, useful to identify the active state of the infection in cats.

Acute-phase proteins (APPs) are increasingly used as tools for the detection of inflammatory diseases in veterinary clinical practice. Use of APP profiles involving at least a major protein (proteins that increase 10-100-fold during the inflammatory response), a moderate protein (increase two-10 fold) and a negative protein (fall in concentration) is highly recommended to differentiate between pathologic states[22]. APPs are species-specific; in cats, Serum Amyloid A (SAA) is a major acute protein, Haptoglobin (Hp) is a positive moderate one, and albumin and paraoxonase-1 (PON1)[22-26] are negatives ones, with the latter also considered a biomarker of oxidative stress[27]. Several investigations have been carried out concerning the usefulness of APPs in infectious diseases in cats, providing valuable information[28-30].

While it is the relationship that may exist between APPs and leptospirosis is not yet known in this species, in dogs, a study reported the C reactive protein-Haptoglobin ratio (CRP/Hp) and albumin serum levels as outcome predictors of *Leptospira interrogans* Australis serogroup infection[31].

Biomarkers of redox status have been studied in cats since they may be contributors for morbidity in many diseases[30,32-35]. Oxidative stress is defined as reactive oxygen species (ROS) over antioxidant defence mechanisms. It can result from an excess of ROS, a reduction in antioxidants, or both[35]. The measurement of the redox status in companion animals has been addressed with novel approaches in the last few years using the TAC assay[35-36]. TAC represents the sum of the activities of the different antioxidants and the antioxidative effects provided by the interactions between individual antioxidants[37-38], also used to evaluate the antioxidant response. Cats seem to be more susceptible to oxidative stress and damage, probably due to the presence of eight reactive and fragile sulfhydryl groups on its haemoglobin molecule and to the particular spleen structure of the species[39-40].

The objective of this paper was to evaluate a panel of acute Phase Proteins (APPs: Serum Amyloid A –SAA, Haptoglobin –Hp, Paraoxonase1 -PON1 and Albumin) and Total Antioxidant Capacity -TAC in free-roaming cats naturally infected by pathogenic leptospires using the principal component method (PCA).

Materials and methods

Selection of cases

Thirty-two serum samples from domestic short-haired cats collected during a previous study on *Leptospira spp.* prevalence in cats in Spain were used in the current study. Serum samples were stored at -80ºC until analysis. Samples were divided into three groups: Group 1 *n* = 8, positive to anti-leptospiral antibodies

detected by MAT (the panel test included 27 serovars, belonging to 20 serogroups and 8 species of *Leptospira*); Group 2 *n* = 5, Positive leptospiral DNA detected by PCR in blood or urine and Group 3 or control group *n* = 19, sera from leptospires-free cats. The absence of *Leptospira spp.* infection in the animals of this group was verified by serology (negative antibodies against *Leptospira spp.* by MAT) and PCR of blood and urine (negative leptospiral DNA amplification). All animals in the study *n* = 32, were tested against feline immunodeficiency and leukaemia viruses (SNAP FIV/FeLV Combo Test®). According to the medical record, all animals in the present study were free-roaming cats and had unremarkable findings upon physical exam.

The study was approved by the Commission of the Doctorate Program in Medicine and Animal Health of the Autonomous University of Barcelona (UAB), approval CEEAH, code 2939

APPs analysis

SAA: Serum amyloid A concentrations were determined by a human turbidimetric immunoassay (LZ-SAA; Eiken Chemical Co., Tokyo, Japan), adapted to an automated analyser (Olympus 2700). This method had been previously validated for use in cats[41]. Serum concentrations lower than 5 µg/ml were considered normal for cats; the limit of detection was set at 0.38 µg/ml[42].

Hp: Serum Haptoglobin concentrations were determined by the use of the haemoglobin-binding method with a commercial kit (Tridelta Development Ltd., Brey, Ireland). The method was previously validated for use in cats[43]. Serum concentrations lower than 3 g/L were considered normal; the limit of detection considered was 0.0088 g/L.

Albumin: Serum albumin was determined using a commercially available kit (Albumin OSR 6102; Olympus Life and Material Science Europe GmbH, Irish branch, Ennis, Ireland) following instructions of the manufacturer.

PON1. Serum PON1 activity was determined by measuring the hydrolysis of p-nitrophenyl acetate to p-nitrophenol, following a previously described method[44], validated in cats[43]. The rate of formation of *p*-nitrophenol was determined at 405 nm after 250 seconds in an automated chemistry analyser (Olympus 2700). The limit of detection was 0.3 IU/ml. Serum concentrations between 3.8 to 7.3 IU/ml were considered normal for cats[27,43].

Antioxidant analysis

TAC: Total antioxidant capacity was determined by the assay Trolox equivalent antioxidant capacity (TEAC1). It is based on the principle that when ABTS (2,2'-azino-bis (3-ethylbenzo-thiazoline-6-sulfonate acid) is incubated with a proper chemical an ABTS radical (ABTS*) is formed, previously described for humans[36] and validated for cats[27]. Serum concentrations higher than 0.35 mmol/L were considered normal; the limit of detection considered was 0.02 mmol/L.

All acute phase proteins and antioxidant analyses were performed on an automated biochemistry analyser (Olympus AU600, Olympus Diagnostic, GmbH).

Statistical analysis

Principal component analysis (PCA) is a dimensionality reduction technique using a linear transformation applied to multidimensional data. The original set of variables (e.g., our set of inflammatory markers, the antioxidant biomarker and antibody titres against *Leptospira spp.*), is reduced to a smaller number of derived variables that may be readily visualised in 2- or 3- principal components containing the highest observed variance. These derived variables can then be compared between categorical variables (e.g., *Leptospira spp.* infection status), through a Student t-test. Median, SD, interquartile range, and range (min-max) of our quantitative variables were also calculated. PCA analysis was performed using the libraries "car" 3-0-6 version[45] "FactomineR" 2.2 version[46], "factoextra" 1.0.6 version[47] and "ggplot2" 3.2.1 version[48] of the R Statistical software 3.6.2 version[49].

Results

Baseline characteristics of the cats

The 32 serum samples included in the study comprised 11 females and 21 males between 6 months to 12.5 years old. The final distribution in the groups was 1 female and 7 males (ranging from 6 months to 7 years old) in Group 1 (anti-leptospiral antibodies); 3 females and 2 males (ranging from 6 months to 1 year old) in Group 2 (positive *Leptospira spp.* DNA in blood or urine) and 7 females and 12 males (ranging from 6 months to 12.5 years old) in Group 3 (leptospires-free cats or control group). Only one cat, included in Group 1, was FIV positive;

all others were negative for both FIV/FeLV. More details about the distribution of the groups and the involved serovars (Group 1) are shown in Table 1.

Table 1. Distribution of groups and leptospiral serovars involved in cats naturally infected by leptospires and leptospires-free cats.

		Total population *n*: 32	**Group 1 *n*: 8**	**Group 2 *n*: 5**	**Group 3 *n*: 19**
Gender	Male	21	7	2	12
	Female	11	1	3	7
Age (y.o.)	Mean	2.6	2.7	0.63	3.0
	Min-Max	0.5-12.5	0.5-7	0.5-1	0.5-12.5
	SD	3	2.43	0.21	3.46
Infecting leptospiral serovars	**Cat**	**Gender**	**Serovar and titre**	**Seronegative**	**Seronegative**
	1	Female	Ballum 1:20		
	2*	Male	Bratislava 1:20 Cynopteri 1:20		
	3	Male	Cynopteri 1:20		
	4	Male	Cynopteri 1:20		
	5	Male	Cynopteri 1:40		
	6	Male	Ballum 1:20		
	7*	Male	Pomona 1:20 Proechimys 1:20		
	8	Male	Sejroe 1:20		

y.o.= years old, (group 1) = anti-leptospiral antibodies (group 2) = positive *Leptospira spp.* DNA in blood or urine, (group 3) = leptospires-free cats or control group, *Cat positive to more than one serovar.

Serum concentrations of APPs, TAC and antibody titre against *Leptospira spp.* obtained in the different groups of the study are shown in Table 2. No concentrations lower than the limit of detection for any of the APPs and TAC studied were obtained.

Table 2. Serum concentrations of APPs, TAC and anti-leptospiral antibody titre. (mean, minimum and maximum and IQR) in cats naturally infected by leptospires (Groups 1 and 2) and leptospires-free (Group 3).

	SAA (µg/ml)	**Hp (g/l)**	**Albumin (g/l)**	**PON1 (IU/ml)**	**TAC (mmol/l)**	**Antibody titre**
Total cats n = 32	8.30	3.84	30.3	4.63	0.58	5.62
MEAN						
MIN-MAX	0.1-130.8	1.64-8.11	21.2-44.9	0.14-7.41	0.43-0.79	0-40
IQR	1.32	1.63	5	1.95	0.14	5.0
Group 1 *n* = 8	0.62	4.05	28.5	3.91	0.63	22.5
MEAN						
MIN-MAX	0.10-1.60	2.49-6.29	23.1-32.9	1.87-6.02	0.46-0.79	20-40
IQR	0.82	1.23	5.3	1.33	0.15	0
Group 2 *n* = 5	46.68	4.47	26.9	3.05	0.53	0
MEAN						
MIN-MAX	0.1-130.8	1.97-8.11	21.2-32.2	0.14-4.84	0.43-0.70	0
IQR	101.9	2.03	5.2	0.86	0.1	0
Group 3 *n* =19	1.43	3.59	32	5.35	0.57	0
MEAN						
MIN-MAX	0.10-6.00	1.64-4.89	24.8-44.9	3.40-7.41	0.44-0.74	0
IQR	1.25	1.68	4.4	1.43	0.12	0

Min = Minimum, Max = Maximum, IQR= Interquartile range, SAA= serum amyloid A, Hp= haptoglobin, PON1= Paraoxonase1, TAC= Total antioxidant capacity, (group 1) = anti-leptospiral antibodies (group 2) = positive *Leptospira spp.* DNA in blood or urine, (group 3) = leptospires-free cats or control group.

Principal component analysis

Our PCA retained two principal components (PC1 and PC2), explaining 60.1% of the observed variability of our set of inflammatory proteins and antioxidant biomarker. PC1 and PC2 accounted for 35.2% and 24.9% of the observed variance and were mainly associated with the inflammatory process and the antioxidant response, respectively (Table 3 and Fig. 1). The most contributing variables in the PC1 were albumin, SAA, HP, and PON1, whereas anti-leptospiral antibodies and serum concentration of TAC did not contribute significantly to the first dimension of our PCA (Table 3). Regarding PC2, anti-leptospiral antibodies and the total antioxidant capacity were the essential variables in terms of contribution to the component (Table 3 and Fig. 2).

The presence of pathogenic *Leptospira spp.* DNA in blood or urine (positive or negative) was a supplementary variable significantly related to PC1 but not to PC2 (Table 3). On the other hand, the presence of antibodies against *Leptospira spp.* by MAT (positive or negative) was a supplementary variable correlated to PC2 but not to PC1 (Table 3).

Table 3. Contribution of variables to PCA and dimensions description

	PC1			PC2		
Active variables	**Contribution** (%)	**Correlation**	***P*-value**	**Contribution** (%)	**Correlation**	***P*-value**
Albumin	**27.46**	0.76	4.07e-07	0.28	-0.06	> 0.05
SAA	**24.71**	-0.72	2.9e-06	11.72	-0.42	1.7e-02
Hp	**21.56**	-0.67	2.26e-05	0.16	0.05	> 0.05
PON1	20.69	0.66	3.71e-05	4.38	-0.26	> 0.05
Antibody titre	3.07	-0.25	> 0.05	**48.41**	0.85	7.64e-10
TAC	2.51	0.23	> 0.05	**34.05**	0.72	2.95e-06
Supplementary variables	**Contribution** (%)	**Correlation**	***P*-value**	**Contribution** (%)	**Correlation**	***P*-value**
MAT (+/-)	na	na	> 0.05	n/a	0.62	0.005
PCR (+/-)	na	0.22	0.005	n/a	> 0.05	> 0.05

Contribution of the inflammatory biomarkers (Albumin, Hp and SAA and PON1), antioxidant biomarker (TAC) and anti-leptospiral antibodies (titre) to the first (PC1) and second (PC2) dimensions of principal component analysis, exploring the response of cats naturally infected by pathogenic leptospires and leptospires-free cats. The outcomes of the MAT test (+/-) and the amplification of pathogenic *Leptospira spp.* DNA through a PCR analysis (+/-) were considered supplementary variables, i.e., not considered for the construction of the factorial axes but used to test statistical differences in the PCA scores. The “na” acronym indicates not applicable**.**

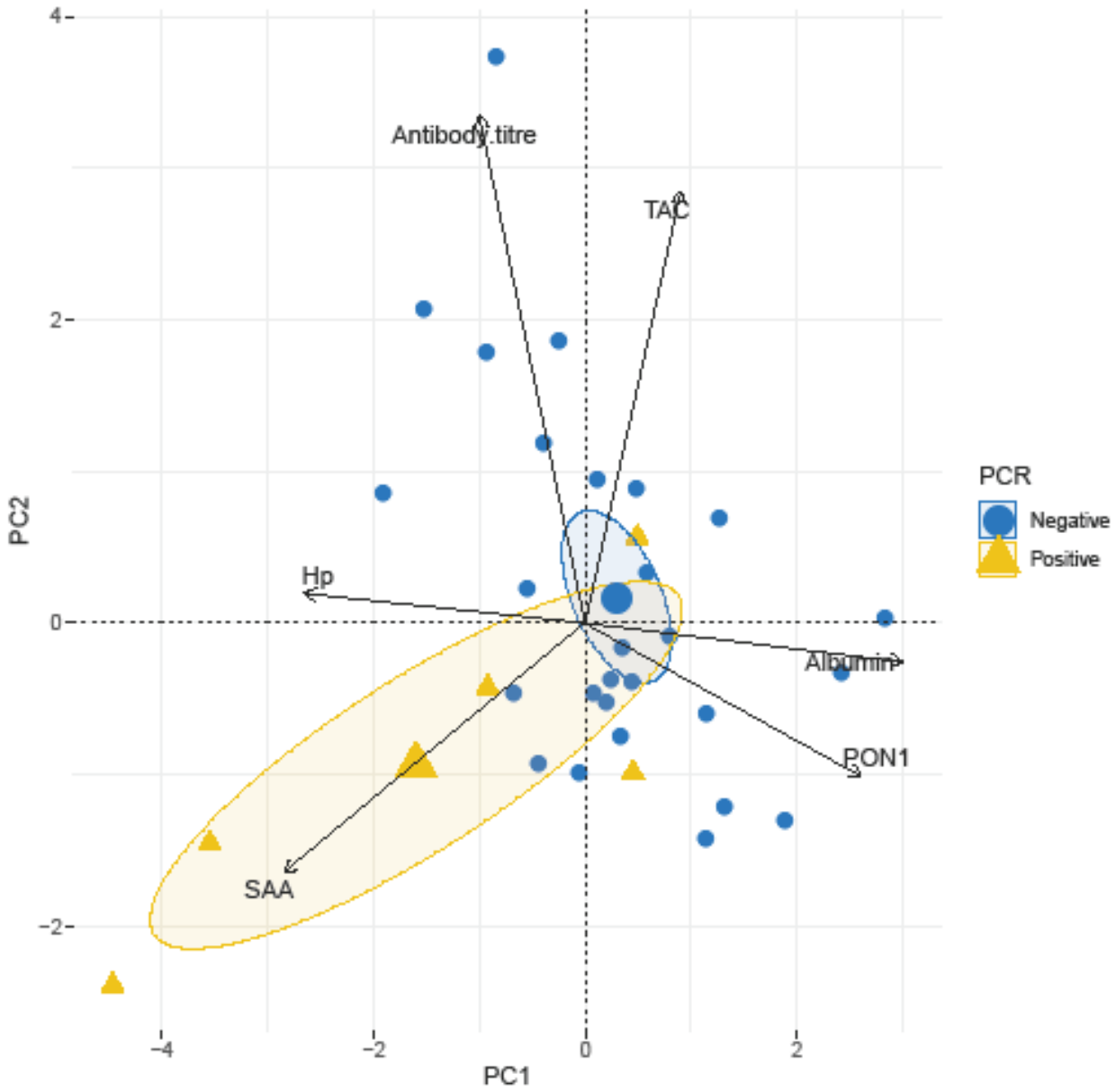

Figure 1. Biplot representing the relationships between the PCR outcome of the pathogenic leptospires (positive/negative), and PC1 and PC2 dimensions of a PCA exploring the relationships among inflammatory biomarkers (Albumin, PON1, HP and SAA), an antioxidant biomarker (TAC), and antibody level against *Leptospira spp.* (antibody titre) in 13 cats naturally infected by pathogenic leptospires and 19 leptospires-free cats from Spain. Neither TAC (total antioxidant capacity) nor the antibody titres against *Leptospira spp.* contributed to the PC1.

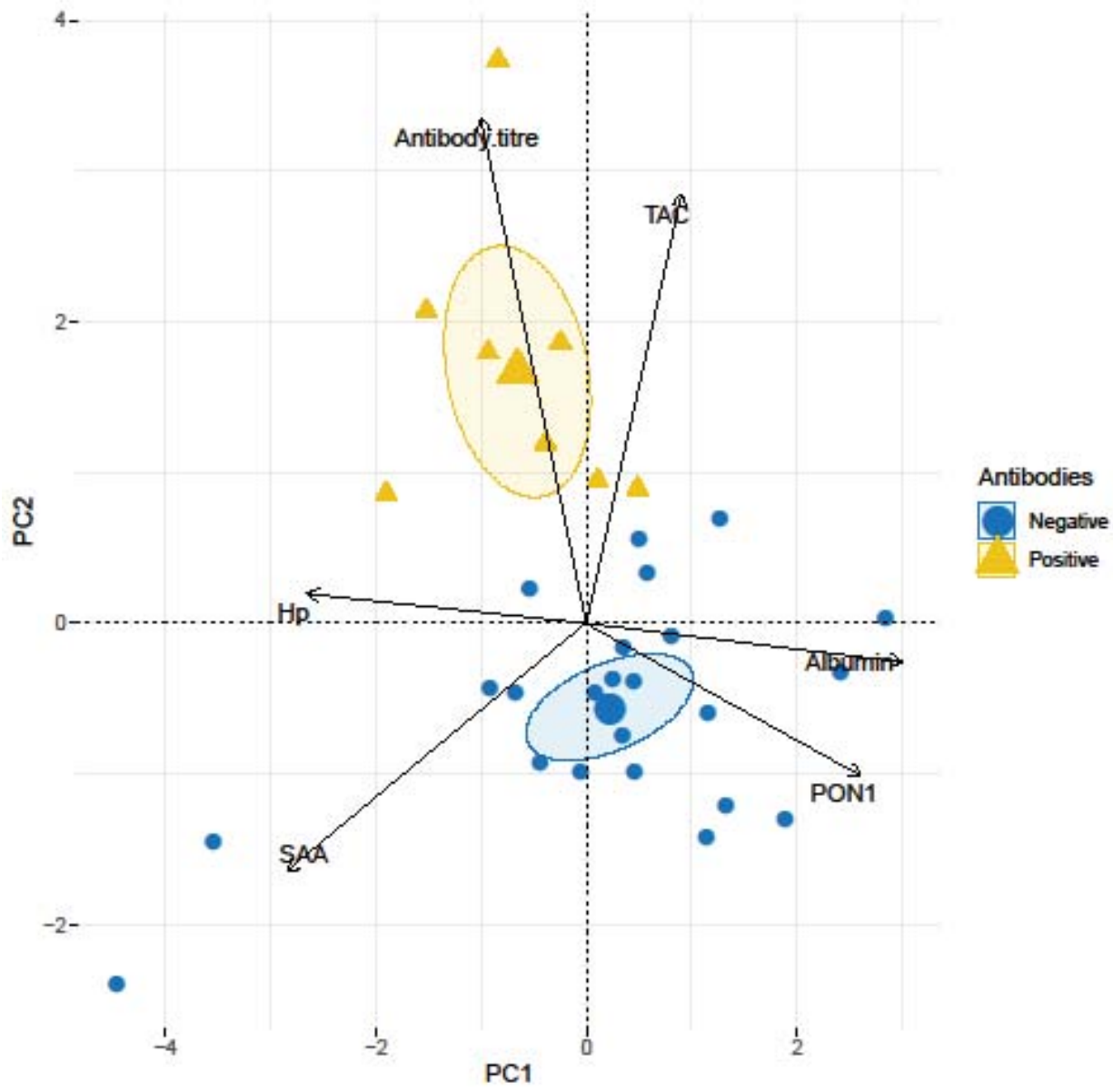

Figure 2. Biplot representing the relationships between MAT outcome (positive/negative), and PC1 and PC2 dimensions of a PCA exploring the relationships among inflammatory biomarkers (Albumin, PON1, HP and SAA), an antioxidant biomarker (TAC), and antibody level against *Leptospira spp.* (antibody titre) in 13 cats naturally infected by pathogenic leptospires and 19 leptospires-free cats from Spain. Inflammatory biomarkers did not contribute to PC2.

Discussion

It is known that cats can sporadically suffer from leptospirosis[12,16], but while the clinical presentation of the disease is rare, it may be missed[2]. Asymptomatic cats can shed the bacteria through urine[9,15], and unlike infected dogs, antibody titres against *Leptospira spp.* detected in sub-clinically infected animals are low in the species[6-7,19]. Cats infected by *Leptospira spp*. may become an incidental or reservoir host. The incidental host develops an acute disease state with mild to moderate clinical signs, leptospiraemia (1-7 days/sub-acute state), leptospires urinary shedding (10 day-about a month/late acute state) and antibodies titres. Reservoir cats usually exhibit a chronic state of infection and have no clinical signs of disease or leptospiraemia, but they do have long-term and intermittent leptospires urinary shedding and low or absent antibodies titres[21].

APPs analysis has been studied in cats with infectious diseases[27-28, 50-51] and its use is highly recommended to determine the active state of disease. TAC as an antioxidant biomarker can contribute to the morbidity of many diseases and is likely to become, along with APPs, an important component in the active state detection of diseases, including leptospirosis in cats.

A PCA statistical approach is based on the fact that cats naturally infected by *Leptospira spp.* may be grouped into different patterns that may be associated with different serum concentrations of APPs and TAC as antioxidant biomarkers. The information obtained could facilitate the understanding of the pathogenesis of *Leptospira spp.* and infection course in cats.

PC1, accounting for 35.23% of the data variance, was significantly associated with the APPs profile or inflammatory pattern. The supplementary qualitative

variable PCR (negative/positive) was significant in the PC1 (R^2 0.22, *P*-value 0.006), suggesting a direct relationship between the presence of the bacteria in blood or urine and the APPs responses.

Patterns of inflammation described in preliminary works were determined and confirmed by our PCA analysis in PCR positive cats (blood or urine). They include a positive correlation found between SAA and Hp (-0.72 and -0.67 respectively), albumin and PON1 (0.76 and 0.66 respectively)[30,52], and negative correlation between SAA-Hp and albumin-PON1[52-53]. It is well known that albumin falls gradually with extensive inflammation, with the reduction in concentration being more noticeable in chronic inflammatory disease[54]. Serum PON1activity was associated with a negative APP and did not play a role as a biomarker of oxidative status in PC1 or in PC2, in contrast to previous studies[27,55].

Likewise, based on *Leptospira spp*. pathogenesis, it is possible to assume that PCR positive animals had a sub-acute infection status (*Leptospira spp.* present in blood/ early stage of the infection) or were mostly were reservoirs (*Leptospira spp.* present in urine/intermittent urinary shedding). Based on the PC1 results, this group of animals (group 2) reflected an active chronic infection, in contrast to those infected with anti-leptospiral antibodies (group 1), which did not reflect an active state of infection. The difference in inflammatory patterns between the two groups of infected cats its very likely due to differences in bacterial load and the infecting serovar involved, among others. In diseases such as FIV, the relationship between antigenic load and high serum levels of APPs, and more specifically SAA, has been shown[56]. Likewise, *Leptospira* serovars involved in the infection have also been reported to influence the inflammatory response in other species. In dogs, it has been shown that serogroup Pomona[57] and

Icterohaemorrhagiae[2] trigger the strongest inflammatory responses and have the worst prognosis. In cats, this remains poorly understood[21]. In our study, the infecting serogroup was not identified in the PCR positive cats as they had no anti-leptospiral antibodies.

PC2, accounting for 24.9% of the data variance, was significantly associated with the oxidative status pattern. A positive correlation was found between antibody titre and serum concentration of TAC (0.85 and 0.72, respectively); thus, as antibody titres against *Leptospira spp.* increased, endogenous antioxidant synthesis increased as well. Likewise, the supplementary qualitative variable MAT (+/-) was significant in the PC2 (R^2 0.62, *P*-value < 0.05).

Cats with low anti-leptospiral antibodies titre (ranging from 1:20 1:40), probably at an early and/or resolution stage of infection, had increases in serum concentrations of TAC, most likely, to counteract the oxidative state associated with the inflammation. In human and companion animals, it has been reported that high serum concentrations of TAC are due to counteracting the increases in oxidants, while decreases are attributed to a persistent state of oxidative stress[27,38, 58].

Seropositive cats seemed to be at risk of developing oxidative stress as antioxidant response measured by the TEC1 assay was above the cut-off point, unlike DNA positive animals, who did not have high TAC values. Antibody titres, in which the antioxidants become exhausted, showing a decrease in serum concentration of TAC and leading to an oxidative status[30], are not yet established. The little information available for cats, has used serum concentrations of TAC to assess the antioxidant components globally. Some authors have reported a

decrease in serum concentrations of TAC associated with oxidative stress[27, 30, 55, 59].

Despite the positive correlation found in our study between antibody titres against *Leptospira spp.* and serum concentrations of TAC, it is not possible to establish whether the variation in the serum concentration of TAC obtained in the two groups of animals naturally infected by *Leptospira spp.* (Group 1 and 2) is due to the low inflammatory response caused by the infection cause or whether this is due to the differences in the available assays for measuring TAC[55].

One of the limitations of our study is the small sample size (total and by groups), and the lack of follow up of the serum values of the biomarkers measured, due to the nature of the animals in the study.

Further studies should be undertaken to elucidate, the *Leptospira* serovars role in cats and their involvement in the inflammatory response through serum concentrations of APPs and TAC as oxidative stress markers by using different assays and enzymes implied in the antioxidant response. To the authors' knowledge, this is the first report measuring the serum concentration of APPs and TAC in cats naturally infected by pathogenic leptospires.

Finally, based on PCA analysis results and the PON1 arrangement on the inflammatory PC1, further experimental studies are needed to estimate its importance as a negative APP in cats.

Conclusions

The use of APPs in *Leptospira spp*. DNA positive cats, helps to identify the active state of infection in sub-acute as well as chronically infected animals.

Leptospira spp. DNA positive, chronically infected animals, are not at risk of developing oxidative stress.

Cats with anti-leptospiral antibodies did not reflect an active inflammatory pattern but instead indicate an antioxidant response in this infection, which is proportional to the antibody titre.

To conclude, this study provides additional information to the limited data available on the use of acute phase proteins and TAC in cats naturally infected by leptospires, which could be helpful for the understanding and diagnosis of leptospirosis in cats.

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5. GENERAL DISCUSSION

A wide variety of animal's hosts including rodents (considered as the main reservoir), domestic mammals, including cats[1] livestock and wildlife, can serve as *Leptospira spp*. infection reservoirs[2].

This thesis provides significant knowledge on the epidemiology of leptospirosis in cats in Spain and on the most common serovars involved in the country. *Leptospira spp.* infection in domestic cats has been described for more than a decade[3] and in recent years there has been growing attention as a reservoir animal[4-7].

Although domestic mammals play a key role in the transmission of the disease, in Spain the cat had not been previously assessed as a possible incidental and/or reservoir host of the infection.

Differences in the epidemiology and the implied serovars among cats by geographical areas have been widely reported[4,8-9], even so, in Spain there was a lack of information in the field. As seen in the **study I** the prevalence of anti-leptospiral antibodies in free-roaming cats in Spain was lower compared to European countries like Germany (17.9%)[5] and Switzerland (10.3%)[10]. In these countries, climatic conditions for the survival of leptospires are less suitable, and the nature of the cat population sampled different (owned cats that attended the veterinary service) compared to this study.

Moreover, the prevalence obtained in our is the lowest reported in cats in Europe[5,10-14]. The differences could be associated with the characteristics of the cat's population, climatic conditions, health status and cat living areas (rural or urban). The highest percentage of seropositivity reported in Extremadura compared to Catalonia is associated with an increased presence of livestock

animals in the former region. These results are consistent with previous research on dogs, confirming a *Leptospira spp*. seroprevalence of 22.3% in Catalonia and 28.6% in Extremadura[15]. Interestingly, that study revealed that the serovars belonging to the Australis serogroup with a 14.9%, were the second most prevalent. Cats from **Study I** had a 20% positivity against serovars belonging to that serogroup. The most remarkable result to emerge from the data analysis is that serogroup Australis is one of the most prevalent serogroups among cats as well as dogs in Europe[5, 10, 13,15-17].

The most frequent serovars involved in feline leptospirosis in Europe belong to serogroups Australis, Autumnalis, Ballum, Canicola, Grippotyphosa, Icterohaemorrhagiae, Pomona and Sejroe[18]. The results obtained in the **study I** reveal the presence of serovars from serogroups Australis, Autumnalis, Bataviae, Cynopteri, Grippotyphosa, Pomona and Sejroe, which have not been previously described among cats in Spain. Serovars belonging to the serogroups Ballum and Icterohaemorragiae were previously described in cats in the country[19]. All serogroups found in the study have been previously reported in cats in Europe[5, 10-14]; therefore, the serovars in Spain are in line with those of the geographical region.

It is essential to remark that most epidemiological studies in *Leptospira ssp.,* employ a limited number of serovars, on average 8 (belonging to different serogroups). However, the World Health Organization recommends including a panel containing 19 pathogenic serovars. Using limited panels decreases technical requirements but can reduce sensitivity if the infecting serovar is not on the panel[20].

Study I included 8 species of *Leptospira*, belonging to 20 serogroups, 27 serovars, and 28 strains, consequently, underdiagnosis was less likely. Therefore, the information collected in our work enhances the knowledge of *Leptospira* serogroups circulating in the country, providing valuable epidemiologic and diagnosis information.

The urinary shedding of leptospiral DNA in cats, was first described 8 years ago[1], several studies suggested the domestic cat as a possible reservoir of the bacterium[4, 6, 8], there is now plenty evidence on the subject, and has even been confirmed that cats can shed viable *Leptospira spp*. by urine[7,21-22]. **Study I** confirmed that free-roaming cats in Spain had leptospiraemia and shed leptospires DNA by urine, in low prevalence. **Study I** confirm the presence of a sub-acute presentation of leptospirosis in a cat (amplified DNA PCR in blood). Previous reports that confirmed the positivity of leptospires in blood (by PCR) were mainly case reports describing acute infection, with clinical signs from mild to severe[3,23] or in induced experimental infections in cats[24-26]. Unlike the previous studies, the cat in **Study I** had no clinical signs of disease at the time of sampling, thus proving that the cat was in a subclinical state.

Earlier research reported worldwide overall urinary shedding ranging from 0% to 67.8%[4, 27-28], with a low prevalence in Germany[5]. As previously in antibody prevalence, the differences in percentages are due to features linked to the animals and regions of the studies. **Study I** is the second study conducted in Europe, providing valuable information by confirming the urinary shedding of leptospires among cats in the continent. **Study I** also provided further evidence that cats in Spain are reservoirs of leptospires, as previously reported in other

countries[1, 6, 27]. All this evidence suggests an important role of the domestic cats in the maintenance and transmission of the bacteria.

In this thesis, it was also investigated the clinicopathological changes and the effect on APPs and an antioxidant biomarker, in cats infected by *Leptospira spp*. Naturally *Leptospira spp*. infection in cats has not been addressed before through the CBC, biochemical profile and urine analysis. In cats, considering that the clinical signs associated with leptospires infection are mild or absent, this data may help to identify chronically infected animals better. The findings of **study II** revealed that a cat that amplifies pathogenic leptospires DNA may have non-regenerative anaemia or is likely to developing it. This fact fits with previous reports in cats, describing anaemia in PCR positive animals[5, 27]. Conversely, acute experimental infections in cats, reported no changes in RBC counts[24-26] and according to **Study II**, anaemia is more common in chronic renal reservoir cats, as opposed to acute infections.

Through **study II,** it was possible to established that seropositive (resolution stage of the infection) cats were more likely to have proteinuria. This finding is consistent with recent results describing a seropositive cat with inactive sediment and marked proteinuria[10]. Proteinuria is likely due to the harmful exposure of leptospires to kidney tubular epithelial cells. Preliminary work in this field determined interstitial nephritis and chronic inflammatory infiltrated in chronic carrier cats[19], proving kidney injuries due to *Leptospira spp*. These results are barely distinguishable from those reported in dogs[28-32], which described proteinuria, with acute leptospirosis in about 60% of the cases.

Remarkable results to emerge from the **study II,** is that leucocyte and platelets count in reservoirs cats had no changes, unlike disease infected cats (incidental host), who had alterations in the leukocyte count mainly[5,14,22]. Changes in platelet counts in cats (thrombocytopenia), are not well documented. On the contrary, in dogs and people leukocyte and platelet counts, are the CBC parameters more often disturb in leptospirosis[30, 33-35].

The values of serum urea and creatinine obtained in **study II** also prove that in infected cats by *Leptospira spp*., renal lesions that affect glomerular filtration, were not evident, unlike acute leptospirosis in dogs[29, 31-32].
Study **II** provides additional support to identify *Leptospira spp*. infection and distinguish infection in cats, through ancillary laboratory analyses.

On the other hand, APPs are increasingly becoming useful ancillary diagnostic tools for inflammatory diseases in clinical practice[36]. They vary with inflammation, infection or stress, among other causes, induced by cytokines[37] and consequently, leptospires infection, was expected to alter APPs levels. As seen in **study III**, the serum concentration of APPs activity varies in *Leptospira spp.* positive DNA amplification cats, this is in good agreement with previous research analysing APPs on infectious diseases in cats [38-40]. SAA protein displayed higher intensity in serum values, reasserting it as major APPs in cats[41], even in leptospires infection. However, the overall effect observed in **study III** in APPs profile, despite being significant, was moderate in positive DNA amplification (blood or urine) and mild or non-present in seropositive cats. Increases in Serum SAA, Hp, and decreases in serum albumin concentrations

and PON1 activity, would indicate an active state of the infection, in chronic infected cats (positive DNA).

Serum PON1 activity reflected an inflammatory instead of oxidative status, in *Leptospira spp.* infected cats. These results have been found in people[42-43], and cats[40].

Study III using PCA lends support to the previous statement of analysing APPs altogether[36], rather than individually, to get a better assessment of inflammatory disease.

Biomarkers of redox status have been studied in cats since they may be contributors for morbidity in many diseases[38, 44-45] and leptospires infection could be one of them. The measurement of the redox status in **study III** addressed using TAC assay, revealed that TAC serum concentrations were proportional to the immune response against *Leptospira spp.* and it wasn't related to the positivity of leptospiral DNA by PCR. **Study III** proves that low anti-leptospiral antibody titres triggers the synthesis of endogenous antioxidants measured by TEAC1 assay. Surely, as previously described by researchers, in inflammatory conditions the serum concentrations of antioxidants vary according to the stage of inflammatory disease, increasing in acute stages to counteract the effect of oxidants and decreasing once they have been exhausted by the prolonged and continuous effect of oxidants[38]. **Studies II** and **III** provides handy information for the clinicians, since the importance of healthy cats is underestimated as a possible reservoir due to the lack of, clear clinical signs and clinicopathological changes associated with *Leptospira spp*. infection.

In summary, this thesis adds valuable information, with public health implication, of leptospirosis in cats and contributes to improving the knowledge of the disease, which remains poorly studied compared to dogs.

Based on the results and literature review of this thesis, it is recommended that veterinarians submit samples to a reference laboratory that participates in a quality assurance program overseen by the International Leptospirosis Society[46] to ensure high-quality MAT results.

This research has raised questions in need of further investigation. Regarding epidemiology, certain researchers state that cats do not play a major role in the epidemiology of leptospirosis based on a low prevalence of urinary shedding[47]. Conversely, there is a report of an immunosuppressed cat owner diagnosed with leptospirosis, who was strongly suspected of been infected by her cat[48]. Furthermore, some studies that sequenced *Leptospira spp*. that infected cats identified similarities to *Leptospira spp.,* previously reported in acute infections in humans[7, 47]. Hence, is very likely that the involvement of the cat in the epidemiology of leptospires depends mainly, on the prevalence of the bacterium in the geographical area, so in endemic areas, the cat may play an important role. However, other factors need to be considered, such as the health status of the cat, the immune status of the infected human and the ability of the cat to shed viable and infecting pathogenic leptospires by the urine.

Future work needs to establish whether cats shed by urine viable leptospires, as urinary shedding of DNA from pathogenic leptospires has been proven to date, but evidence of the viability of the urine shed leptospires by bacterial culture in cats is scarce. Further efforts are needed in this field, as leptospires are highly

demanding for their growth and bacterial isolation. Only once the viability of the bacteria will be proven, can a vaccine be considered for use in cats, but not before.

Likewise, knowing that the same serovar can infect different animal species[2] and considering that the Australis serogroup has shown a significant prevalence among dogs and cats in Europe[5,10,13,15-17]. Once the viability of the pathogenic leptospires shed by urine in cats has been proven, further experimental investigations are needed to determine the importance of cats in the persistence of the infection among dog's population.

6. CONCLUSIONS

1. The fact that cats infected with pathogenic leptospires, can shed DNA in their urine, without developing clinical disease and without the development of antibodies, must drive a reflection on the role of cats as reservoirs and possible transmitters of pathogenic *Leptospira spp*. to humans.

2. Laboratories in Spain that perform serological diagnostic tests using the MAT for the diagnosis of feline leptospirosis, in addition to following the statements of the International Leptospirosis Society, must include in their panel serovars belonging to the serogroups reported in this study. This simple recommendation would increase the sensitivity of the test, as the infecting serovar could be on the panel.

3. The simultaneous use of PCR and MAT techniques in leptospires infection, achieves a rapid and accurate diagnosis in cats.

4. Cats naturally infected by pathogenic leptospires can develop non-regenerative anaemia. Those that amplify pathogenic *Leptospira spp.* DNA by PCR (urine or blood), are at higher risk of it, while *Leptospira spp.* seropositive cats, are at higher risk of developing proteinuria.

5. The analysis of the APPs profile by PCA, allowed the detection of the active state of the infection in positive cats to pathogenic leptospires DNA.

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