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New aspects of leptospirosis in shrews and dogs; investigation on the prevalence and pathology

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Abstract

Leptospirosis is a complex and often underestimated global bacterial disease and continues to be of zoonotic concern. It is difficult to diagnose, has an unclear pathogenesis and several new *Leptospira* species of often unknown health impact have been discovered in recent years. Depending on the mammalian species affected and the causing serovar, the clinical disease can range from mild fever to severe internal bleeding, jaundice, and kidney failure. Other animals are subclinical carriers of *Leptospira*, maintaining the spirochaete in renal tissue and shedding it via the urine.

In 2013 a new *Leptospira* species (*L. tipperaryensis)* was discovered in Ireland in greater white-toothed shrews (GWTS - *Crocidura russula*), an invasive species. This study investigates the prevalence and the impact of *L. tipperaryensis* on the health of the host (GWTS), and on the only native shrew species in Ireland, the pygmy shrew (*Sorus minutus*). A further study examines the association of a rarely reported renal pathology, exudative glomerulonephritis, in Swiss dogs and infection with *Leptospira* spp. These evolving new aspects of leptospirosis are evaluated by similar methods using histopathology, immunohistochemistry and PCR assays on renal tissues.

Results showed no prevalence of *Leptospira* sp. in shrews sampled in Ireland in general, and no presence of any significant renal pathology. Therefore, no negative impact of *L. tipperaryensis* on the health and distribution of the GWTS and the pygmy shrew was observed, and onward transmission within the population appears unlikely. Conversely, an association was found between this new fatal renal pathology of exudative glomerulonephritis and *Leptospira* sp. infection in dogs, suggesting an underlying different pathogenesis and possible new serovar.

These studies have added significantly to the knowledge on leptospirosis. Future studies will be aimed in elucidating the pathogenesis of exudative glomerulonephritis and identifying the *Leptospira* species and serovar involved.

Statement of Original Authorship

I hereby certify that the submitted work is my own work, as completed while registered as a candidate for the degree stated on the title page, and I have not obtained a degree elsewhere on the basis of the research presented in the submitted work.

Collaborations

Paper 1

'Exudative glomerulonephritis associated with acute leptospirosis in dogs' published in Veterinary Pathology. First published Oct 29, 2023. doi:10.1177/03009858231207020

Monika Hilbe, Horst Posthaus, Giulia Paternoster, Simone Schuller, Michelle Imlau, Hanne Jahns.

Paper 2

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Michelle Imlau, and Hanne Jahns.

Paper 3

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Michelle Imlau, Mamoun Saeed, Jane Cryan, Séamus Hoey, Myles McKenna, Hanne Jahns, Pamela Kelly

Poster 1

'Retrospective study of *Leptospira* infection in dogs: unusual renal Pathology and diagnostic challenges' presented at UCD College of Health and Agricultural Sciences Graduate Research Student Symposium, May 2023, Dublin, Ireland.

Michelle Imlau, Monika Hilbe, Hanne Jahns.

Poster 2

'Post mortem findings in grey seals (*Halichoerus grypus*) and harbour seals (*Phoca vitulina*) stranded on the Irish coast' presented at Student Summer Research Awards, September 2022, Dublin, Ireland.

Han Zhang, Audrey Saint-Marc, Michelle Imlau, Elena Plamenova, Hanne Jahns.

Conference Presentation 1

'Comparison of multiple imaging modalities for evaluating *ex vivo* adrenal glands in dogs' presented at IVRA EVDI Joint Conference, June 2023, Dublin, Ireland.

Olga Amorós, Michelle Imlau, Eimear Shorten, Antonella Puggioni, Giulia Dalla Serra, Brian Cloak, Séamus Hoey

Conference Presentation 2

'First reports of the lungworms *Troglostrongylus brevior* and *Angiostrongylus chabaudi* in European wildcats (*Felis silvestris*) in Switzerland' presented at Zoo and Wildlife Health Conference, May 2022, Emmen, Netherlands.

Diana S. Gliga, Patrick Scherrer, Michelle Imlau, Francesco C. Origgi, Samoa Zürcher-Giovannini, Caroline F. Frey, Marie-Pierre Ryser-Degiorgis, Walter Basso.

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List of Abbreviations

°C	degrees Celsius
ALT	alanine transaminase
AP	alkaline phosphatase
bp	base pairs
cm	centimetre
CT	cycle threshold
DAB	3,3'-Diaminobenzidine
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
DSH	Domestic short-haired cat
dUTP	deoxyuridine triphosphate
ECM	extra cellular matrix
EEA	European Economic Area
ELISA	enzyme-linked immunosorbent assay
EU	European Union
FAO	Food and Agriculture Organization
FFPE	formalin-fixed paraffin-embedded
Fig	figure
g	gram
GN	glomerulonephritis
GSD	German Shepherd Dog
GWTS	greater white-toothed shrew
HE	haematoxylin and eosin
IF	immunofluorescence
IHC	immunohistochemistry
JRT	Jack Russell Terrier

km²	square kilometre
LPHS	leptospiral pulmonary haemorrhage syndrome
LPS	lipopolysaccharide
MAT	microscopic agglutination test
min	minute
ml	millilitre
N	population size
n (n')	sample size
NA	not available
ng	nanogram
ng/µl	nanogram/microlitre
nm	nanometre
NSF	no significant finding
OIE	Office International des Epizooties
p̂	population proportion
PAS	Periodic acid–Schiff
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	potential of hydrogen
pmol	picomoles
PS	pygmy shrews
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RT	room temperature
s	second
TEM	transmission electron microscopy
UK	United Kingdom
USA	United States of America
UV	ultraviolet
WGS	whole genome sequencing

WOAH	World Organisation for Animal Health
WS	Warthin-Starry
z	z score
ε	margin of error
µg	microgram
µl	microlitre
µM	micromole

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General Introduction

Leptospirosis is a common zoonosis worldwide [1] and affects numerous domestic and wild mammalian species, as well as fish, amphibia, and reptiles [1-4]. Despite the omnipresence of the disease and the constantly evolving knowledge many aspects of leptospirosis are still poorly understood due to epidemiological factors, difficulties in diagnosis, the complexities of host-leptospiral relationships, as well as constantly discovered new species which have an unknown impact and pathology.

To date there are over 300 different serovars, which are classified based on their outer surface structures [5]. Pathogenic serovars cause subclinical to mild disease in well adapted maintenance hosts [5], but incidental hosts experience a variety of severe clinical signs, including fever, icterus, vomiting and diarrhoea, and death [6, 7].

Diagnosing leptospirosis poses numerous challenges. In a clinical setting, serology serves as the gold standard for detecting *Leptospira*. Microagglutination tests (MAT) offer quick results, especially when the appropriate antibodies for the specific serovars are available and used. Very acute disease might be missed with this method however, as antibodies need seven to ten days to rise to detectable levels. Similarly chronic infections without a significant antibody level increase are not suited for MAT. Numerous other direct and indirect methods are available, especially molecular techniques like quantitative polymerase chain reaction (qPCR) and conventional PCR which are becoming increasingly popular [8]. These molecular methods are frequently used when screening wildlife for carrier status where sample availability is limited, and the main goal of investigation is to determine whether animals carry leptospirosis, and not if there are pathological changes present (which are usually absent in carriers). Immunohistochemistry (IHC) provides a satisfying visualisation of chronic carriers when persistent *Leptospira* are maintained in proximal convoluted tubules of the renal cortex. To achieve a post-mortem diagnosis the latter two methods are very suitable. Extracting DNA (deoxyribonucleic acid) is possible from freshly frozen or formalin-fixed paraffin-embedded (FFPE) tissue, with kidney or liver being the preferred tissues. IHC can be used as a complementary or alternative method for detecting *Leptospira* sp. post-mortem.

The annual epidemiological report for 2020 reported 565 confirmed cases of human leptospirosis in 21 European countries [9], which equals a notification rate of 0.14/100,000

population in the EU/EEA. Ireland had a slightly higher notification rate with above 0.50/100,000 population. For comparison highly affected tropical regions report average incidences of 1.9/100,000 population in Brazil [10] and up to 24.5/100,000 population in certain regions of Thailand [11]. Case numbers and incidences for dogs are less thoroughly recorded. Studies investigating healthy dogs in Ireland observed that 7% of urine samples contained pathogenic leptospires [12]. Another study of dogs in Switzerland discovered elevated MAT titres for at least one serovar in 24.9% of the tested samples. In the Netherlands however, only 2 to 13 dogs per year were diagnosed with leptospirosis from 2010 to 2014 [13], illustrating the variation in the prevalence of this pathogen.

There is no published data in the last 50 years on the prevalence of *Leptospira* in Irish wildlife [14] except for the recent discovery of a new leptospiral species in greater white-toothed shrews (GWTS - *Crocidura russula*) [15]. In the context of leptospirosis in wildlife species, the focus is often on their role as carriers, with limited attention given to the potential clinical impact of leptospiral infection. Clinical leptospiral disease has been documented in numerous wildlife species, including cervids [16], camelids [17], mustelids (sea otter) [18], procyonids (raccoons) [19], pinnipeds (seals) [20], monkeys [21], rhinos [22], marsupials and monotremes [23], manatees [24], and rodents (beaver) [25]. Wildlife presents a similar spectrum of clinical disease to domestic animals, from mild clinical signs to fatal outcomes depending on the host-serovar interaction. Rodents, particularly rats (*Rattus* sp.), are considered the primary reservoir for pathogenic leptospiral serovars and are the main source of environmental contamination, especially water, therefore spreading the infection to humans and other mammals. Serovar Icterohaemorrhagiae is the main serovar involved. Even though carriers are considered to be clinically silent, interstitial nephritis is the predominant lesion in rats and other carriers [26-29], as seen with leptospirosis in general [7, 26, 27]. Other wildlife species identified as carriers for *Leptospira* in Europe are voles and other small rodents that carry serovar Grippotyphosa, and hedgehogs (reservoir hosts for Australis [30, 31]). Studies have detected *Leptospira* spp., mainly *L. kirschneri*, in several shrew species [32-36]. GWTS are an invasive species in Ireland, and it was observed that their expansion was accompanied by local extinction of Ireland's native shrew species, the pygmy shrew (*Sorex minutus*) [37]. Leptospiral isolates were cultured from their urine and initial sequence analysis of *16S* ribosomal RNA and *secY* indicated that the novel isolates were serovars that belong to *L. alstonii.* Subsequent sequencing confirmed them to be a new species, named *Leptospira*

tipperaryensis after the Irish midland county Tipperary [15, 38]. The impact of *L. tipperaryensis* on the health of the GWTS and the pygmy shrew has not yet been investigated. A newly introduced pathogen can lead to unexpected or unusual lesions due to lack of immunity or unknown host-serovar interactions.

The objectives of the first project of this thesis were to explore the impact of *L. tipperaryensis* on the health of the Irish shrew species and examine its role in the local extinction of the native shrews. A combination of IHC with different antibodies and real time/quantitative PCR was applied to detect *Leptospira* sp., and histological examination evaluated inflammatory changes within kidneys.

In small domestic animals, dogs are the most commonly affected species. They suffer from acute disease with fever, haemorrhage, and death, and can develop chronic renal failure [6] mainly from serovars Canicola and Icterohaemorrhagiae. In accordance with this, traditional vaccination for dogs contained inactivated parts of the serovars Icterohaemorrhagiae and Canicola. This has been recently updated to a multivalent vaccine inducing immunity against Canicola, Icterohaemorrhagiae, Bratislava, and Grippotyphosa [39].

Recognising classic pathological changes is a valuable tool when diagnosing leptospirosis, however leptospirosis is an unpredictable disease with a changing distribution of serovars and new serovars or species being continuously discovered. Infection with *Leptospira* mainly causes injury to the kidneys, liver, and vasculature. This results in acute neutrophilic tubulointerstitial nephritis with tubular degeneration and necrosis, or chronic lymphoplasmacytic to histiocytic tubulointerstitial nephritis with cortical fibrosis and tubular atrophy. Hepatic lesions include a very characteristic (but not unique, i.e. pathognomonic) dissociation of hepatocytes and further portal lymphocytic inflammation with hepatocellular degeneration and possible evidence of cholestasis. Affected vessels display necrotising vasculitis and fibrinoid changes [7]. Over the past 20 years an unusual renal lesion, an exudative glomerulonephritis (GN), was observed with increased frequency in Swiss dogs. These dogs had been clinical suspects of leptospirosis and an association with this pathogen was suspected. GN is not common in veterinary medicine, except in the viral caused porcine dermatitis and nephropathy syndrome [7, 40, 41], and is not a classic feature of leptospirosis. Furthermore, GN in veterinary medicine is primarily a lesion associated with circulating immunocomplexes or complement. The second study of this thesis investigates the

association with this new rare exudative GN and *Leptospira* sp. infection. Similar to the shrew study different techniques (IHC, qPCR) were applied to detect *Leptospira* in renal tissues.

Both studies used similar diagnostic methods and histopathology to investigate new aspects of leptospirosis.

The objectives of this thesis are:

- Investigate of the prevalence of the new species *Leptospira tipperaryensis* in GWTS and pygmy shrews
- Evaluate the impact of *L. tipperaryensis* on the health of GWTS
- Explore the role of *L. tipperaryensis* in the local extinction of pygmy shrews
- Investigate the association of exudative GN in dogs and *Leptospira* sp. infection

Chapter 1

Literature Review

1. *Leptospira* sp.

a) Species and Serovar

Leptospira sp. are bacteria belonging to the phylum and class of Spirochaetes and more specifically to the order of Leptospirales and the family *Leptospiraceae*. The current accepted taxonomy is according to the List of Prokaryotic names with Standing in Nomenclature (LPSN) and the National Center for Biotechnology Information (NCBI), and the phylogeny is based on 16S ribosomal ribonucleic acid (RNA) analysis by 'The All-Species Living Tree' Project (Release LTPs123) [42].

The genus *Leptospira* comprises over 60 species categorised into pathogens, saprophytes, or intermediate groups [5, 43, 44]. Those *Leptospira* species are further classified into >300 serovars, of which closely related serovars are grouped into serogroups [43]. The serovar classification of *Leptospira* is based on the expression of surface components, i.e. lipopolysaccharide (LPS) antigens. However, neither serogroup nor serovar reliably predicts the species of *Leptospira* because the same serogroup (i.e. share common antigenic determinants) or serovar (i.e. antigenically indistinguishable isolates) may occur within two or more species. That means that in practice genetically unrelated *Leptospira* can be antigenically identical [7].

The serovars that domestic and wild animals are exposed to vary between countries, and even within countries between different regions. The majority of pathogenic serovars that are found with a global distribution belong to the species *L. interrogans, L. borgpetersenii,* and *L. kirschneri* [8]. The main serovars causing disease in (mainland) European dogs are Icterohaemorrhagiae, Grippotyphosa, Australis, Sejroe, and Canicola. The Pomona serogroup for instance, presents an important role in North America but is rare in Europe [39]. In Ireland the most common serovars are Icterohaemorrhagiae and Hardjo [45].

b) Morphology

Leptospires are about 0.1 µm in diameter by 6–20 µm in length with distinctive hooked ends like a question mark (Fig. 1), which originally led to the name *L. interrogans*. Like all spirochaetes they obtain a classic double membrane in which the outer membrane presents the LPS, the main antigen for *Leptospira* [46], which is structurally and immunologically similar to lipopolysaccharides (LPS) from Gram negative organisms. In addition to LPS, structural and functional proteins form part of the leptospiral outer membrane. A large proportion of such

proteins are lipoproteins with relative abundance on the cell surface: LipL32 > LipL21 > LipL41 [47].

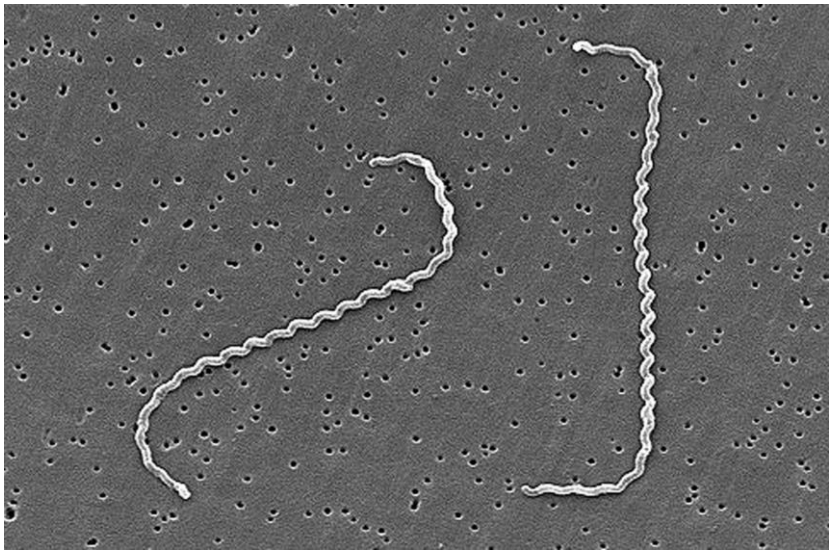

Figure 1. Scanning electron micrograph of *Leptospira interrogans* isolated in 1915 clearly demonstrates the characteristic hooked ends. (Image obtained from the Public Health Image Library at the Centers for Disease Control and Prevention; content provider CDC/NCID/Rob Weyant; Photo Credit Janice Haney Carr).

c) Pathogenic vs. Apathogenic

At the beginning of the 20th century *Leptospira* was first described as being "Found in Yellow-Fever Tissue" [48] and since then the genus *Leptospira* has been traditionally divided based on its virulence into two groups, saprophytes, i.e. *Leptospira biflexa* sensu lato, and pathogens, i.e. *Leptospira interrogans* sensu lato. Later, phylogenetic analysis showed that *Leptospira* can be divided further, adding a third lineage according to their pathogenic effects: saprophytic, intermediate, and pathogenic, which in total comprised about 35 species at that time [1, 43, 49]. Recent studies undertook a long term, systematic and worldwide examination of existing and potential new *Leptospira* species and discovered 30 novel species of *Leptospira* which led to a new classification system: two major clades of saprophytes and pathogens which, based on phylogenetic analysis, are further subdivided into two subclades each: subclade P1 (formerly known as the pathogen group), P2 (formerly known as the intermediate group), S1 (formerly known as the saprophyte group) and S2 (a new subclade with mostly new species) [5]. Today there are 65 different *Leptospira* species that include more than 300 serovars [5, 43, 44, 50] and it is likely that new species will continue to be discovered in the future. According to the WHO classification, saprophytes (i.e. apathogenic

Leptospira) need to fulfil the following criteria: growth at 13 °C in the presence of 8-azaguanine plus conversion into a spherical form in 1M NaCl. Conversely pathogenic *Leptospira* isolates contain LipL32 and do not grow in presence of 8-azagunaine [51]. Additional tools for the classification of pathogenic *Leptospira* include examination of DNA-DNA relatedness, 16S rDNA and their *secY* sequence [38].

d) Host Adaption and Cycle of Infection

Leptospirosis is an important systemic disease in humans and domestic animals caused by serovars of *L. interrogans* (sensu lato). It is especially important as an abortive agent in farm animals and it causes acute diseases, characterised by fever, renal and hepatic insufficiency, and pulmonary manifestations mainly in dogs, cattle, and swine [7]. Each serovar is specifically adapted to "maintenance" hosts in which infection is mainly subclinical. Pathogenic leptospires are maintained in the proximal convoluted tubules of the kidney and in some maintenance hosts like cattle, sheep, and pigs, also found in the genital tract [52]. Maintenance hosts (sometimes also called reservoir hosts) are in general more susceptible to an infection by the respective serovar although the pathogenicity is lower. The persistence of *Leptospira* within the tissue of maintenance hosts mentioned above is a classical and important feature to maintain the *Leptospira* infection cycle. Vaccination is the method of choice to protect domestic animals from leptospirosis, but choosing appropriate antigens in the vaccine in terms of relevance to the animal species and country or region specific serovars is of utmost importance [53]. The immune response varies amongst animals and as such it is not surprising that the antibody response following vaccination can exhibit variability in certain animals, such as dogs, which presents challenges for vaccination in this species. Dogs, as in humans and pigs, develop a predominantly humoral (antibody) mediated immunity against leptospirosis, and when comparing MAT results in dogs after receiving multivalent vaccines (targeting Canicola, Grippotyphosa, Icterohaemorrhagiae, and Pomona) a variation in maximal titres, the serovars inducing the maximal titres, and the time required to reach maximal titres can be observed [7, 54]. In contrast, mechanisms of immunity in cattle are correlated with a Th1 response mediated by interferon gamma release [55]. This means that even cattle with high levels of serum antibody levels are not protected from natural infection. Nevertheless, cattle are reported to build a sufficient and protective immunity against *Leptospira* after the use of monovalent products, but some multivalent products only

stimulated poor immunity [8, 56]. The following factors need to be considered when designing a vaccination regime: vaccination before first exposure (to avoid carrier status or chronic infection), yearly repetition (to maintain a sufficient immunity) and in general a tailored choice of vaccine regarding target population (animal species) and current epidemiologic situation (prevalence of serovars in the respective region) [8]. Nearly all mammals and marsupials worldwide are proven to be potential carriers of leptospires. Rodents, especially rats (*Rattus* sp.), are considered the most relevant reservoir of pathogenic leptospiral serovars, more specifically serovar Icterohaemorrhagiae. To a lesser extent voles and other small rodents carry serovar Grippotyphosa, and hedgehogs are reservoirs for Australis in Europe [30]. Other common carriers include small marsupials, cattle, pigs, and dogs [1]. Maintenance hosts mostly transmit *Leptospira* directly via urine, postabortion discharges, milk, or through venereal or transplacental transmission. *Leptospira* can survive weeks to months in muddy conditions or stagnant water and occurs especially in autumn (temperate climate) or winter (tropical regions). Even well adapted *Leptospira* sp. can trigger diseases in other species, which are termed "incidental" hosts. Incidental hosts often then distribute *Leptospira* via their urine into the environment so that infection occurs indirectly.

2. Diagnosis

Laboratory tests for diagnosing *Leptospira* can be distinguished between direct testing, i.e. detecting leptospires, leptospiral antigens, or leptospiral nucleic acids; and indirect testing, i.e. detecting anti-leptospiral antibodies. Regarding potential material to test for leptospirosis, several organs (e.g. liver, lung, brain, and kidney) and body fluids (e.g. blood, milk, cerebrospinal, thoracic and peritoneal fluids) can be used to diagnose an acute clinical disease. For chronically infected animals (not showing any clinical signs), potential material include tissue from the kidney or genital tract, and urine, or if available the foetus in the case of a chronic infection in its mother.

a) Serology

i) Microscopic Agglutination Test

Serological testing can identify specific antibodies in the serum. It is the most commonly used diagnostic tool for leptospirosis, and the MAT is considered the standard serological test [8]. In MAT a panel of live leptospires, relevant to the area where the sample originates, is tested with the serum of the affected person/animal. Different dilutions

(representing the antibody titre) of serum are mixed, incubated and evaluated for agglutination under a dark-field microscope. Dead antigens can be used in MAT, but this lacks specificity. Knowledge of regional circulating *Leptospira* species and serovars affecting the examined species are essential, although MAT aims to detect serogroups rather than a serovar [57]. Another advantage of MAT is that it can be used to test both individual animals and entire herds. It has proven to be particularly useful in testing for acute disease in an individual animal. This requires a detectable rise of antibodies which usually occurs after two to eight days [58, 59] and means that peracute, very early disease can be missed. World Organisation for Animal Health (WOAH) defines a four-fold rise in antibody titres in paired acute and convalescent serum samples as diagnostic and recommends testing at least ten animals or 10% of the herd (whichever is greater) for a meaningful assessment of herd status. Limitations of MAT exist in terms of chronic or endemic herd infections, as in this case MAT titres may be beneath the lowest titre threshold (i.e. 1/100 in final dilution) [8].

ii) Enzyme-Linked Immunosorbent Aassay

For enzyme-linked immunosorbent assay (ELISA), antigens from a sample are mixed with the corresponding antibody which is linked to an enzyme. After incubation any unbound antibodies are removed and the substrate for the linked enzyme is added. The bound enzymes will react with the substrate and cause a detectable signal, usually a colour change. Many ELISAs have been established for the detection of antibodies against *Leptospira,* and it is primarily used for the diagnosis of recent infections and for screening in experimental studies. Furthermore, in cattle an ELISA can be used for health assessment of an individual animal or the herd, e.g. to evaluate levels of infection of serovar Hardjo. This can be achieved by individual testing of blood or milk, or by testing of samples from a bulk milk tank. ELISA is especially recommended in the context of confirming the absence of an infection in populations/herds, to support eradication programs, and to evaluate the immune status in post-vaccination individuals/herds [8]. Commercial kits are available and most commercial ELISA kits use the nonpathogenic *Leptospira biflexa* patoc strain as an antigen. This method is not suitable for identification of the causative serovar or serogroup, and positive ELISA should be confirmed by MAT. ELISA can detect antibodies from day six to eight after infection, which is earlier than the MAT, but likewise, it may show negative results earlier following infection.

b) Culture and Phylogenetic Analysis

Microbial organisms can grow and multiply in a culture by setting them into a specific culture medium under controlled laboratory conditions. Isolating *Leptospira* is very challenging and requires technical experience. Additionally, it should be ensured that no antibiotic residues are present, that the tissue is not autolytic, that it is quickly processed for culture after collection, and if it is a urine sample that the pH is within range. If all these requirements are met, isolating leptospires is a very specific method of demonstrating their presence [8]. Leptospires are obligate aerobes with an optimum growth temperature of 28–30°C. They grow in simple media enriched with vitamins B1 and B12, long-chain fatty acids, and ammonium salts. Growth of leptospires in media containing serum or albumin and in protein-free synthetic media has been described. Several liquid media enriched with rabbit serum were described in the past. Currently, the most widely used medium is based on the oleic acid, bovine serum albumin and polysorbate (Tween) medium EMJH [1]. Contamination of the sample can be very problematic and adding selective agents, e.g. 5-fluorouracil and a variety/combination of antibiotics can help to control contamination [8]. A drawback of adding inhibiting substances is the risk of lowering the chances of finding leptospires if they are only present in small numbers. Additionally, some strains simply do not grow in those selective environments. In general, growth of leptospires is rather slow on primary isolation, and cultures must be retained from 16 up to 26 weeks before being discarded [60]. The best use for isolating and culturing leptospires is for testing for freedom from leptospires in an individual animal prior to movement, and for confirmation of clinical cases.

Subsequently cultured isolates can be phylogenetically analysed, i.e. examined regarding their taxonomic evolutionary history, and their relatedness and lineage. This is not directly associated with diagnostics, but no less important in terms of classification of serovars and understanding their evolution. Additionally, recognising new serovars or serogroups is important for monitoring circulating serovars in geographic regions, recognising shifts in their distribution, or identifying species specific lesions or clinical signs. Some targets of phylogenetic analysis have already been mentioned above. 16S ribosomal RNA is small subunit of a prokaryotic ribosome that due to its slow rate of evolution or mutation has been considered a valuable contribution in reconstructing phylogenies. More recent studies do not favour this analysis, stating it to be highly conserved and not distinguishable enough to differentiate between *Leptospira* serovars [5]. The *secY* gene sequence translates into a

translocase protein, more specifically into its subunit secY and it is considered the main transmembrane subunit of certain bacteria and thus useful for phylogenetic analysis of *Leptospira* [61]. Additionally this could be further expanded to whole genome sequencing, which decodes the organism's entire genetic material. This technique helps track outbreaks, understand virulence factors, investigate antimicrobial resistance, and study the evolutionary relationships between different strains for better understanding of the epidemiology and pathogenesis of leptospirosis.

c) Fluorescent Antibody Test

The fluorescent antibody test (FAT) can be used to quickly detect pathogens in various fresh specimens like tissues, fluids and swabs. It uses antibodies that are labelled with fluorescent chemicals that illuminate and become visible after binding to the target antigen. Fluorescent antibody techniques or immunoperoxidase of the original specimen can be carried out and is a useful diagnostic technique, almost equivalent in sensitivity to isolation in laboratories with specific expertise in this area [57]. One study mentions that autolysis of tissues, contamination and freezing and thawing can significantly reduce its sensitivity [62]. FAT is very labour intensive; it requires specialist training and the maintenance of live cultures only to test for specific serovars, which makes it challenging to obtain this method as a standard in one's laboratory setting. Additionally, some strains are not optimal for detection by FAT, e.g. Bratislava, as the small size of these organisms makes their detection difficult [7].

d) Polymerase Chain Reaction

PCR amplifies rapidly numerous copies of a specific DNA sample, by using several components and reagents. Most importantly this includes DNA polymerase, two DNA primers, and deoxynucleoside triphosphates (dNTPs). DNA polymerase is a heat-resistant enzyme that polymerizes new DNA strands. Its binding tools are the two DNA primers that are complementary to each three prime ends of both DNA strands of the targeted DNA template. Finally, the dNTPs are the building blocks from which the new DNA strand is synthesised. As in other areas, the development of molecular methods for diagnosing *Leptospira* are fast and wide-ranging. These methods are being used more and more for the detection of leptospires in various tissues and body fluids. Considering the actual costs of this technique it is not suitable for systematic herd testing, but can be applied in cases of individual animals, similar to indications for isolating and identification mentioned above. One huge

advantage is the sensitivity of this method and the possibility of a quick diagnosis. Real-time (quantitative) PCR is even faster than regular PCR, and some studies attest to it being less vulnerable to contamination [63]. Hereby two options exist; firstly, aiming for genes that are generally present in *Leptospira* bacteria, e.g. *gryB, rrs* (16S rRNA gene) and *secY*, or secondly, targeting genes which are restricted to pathogenic *Leptospira*, e.g. *lipL21, lipL32, lipL41, ligA* and *ligB* [64, 65]. As in other settings, contamination (especially in clinical samples) can be problematic i.e. false-negative results can occur due to amplification inhibitors via contamination with faeces or by autolysis. Furthermore, according to the WOAH, founded as and formerly known as Office International des Epizooties (OIE), the main issue with using PCR as a diagnostic tool in animal leptospirosis remains its validation [8]. More specifically, each laboratory is responsible for its own validation of the specific assay they use for the material (tissue, fluid) and species being tested. Another modification of the classical PCR method are nested PCRs. Nested PCRs were developed to improve sensitivity and specificity by using two sets of primers and two consecutive PCR reactions [66, 67]. In the first PCR reaction, primers are targeting a sequence upstream from the second set of primers. The amplicons generated in first PCR reaction are used as a template for the second PCR process with the second set of primers. Sensitivity and specificity of DNA amplification can be significantly improved with this method. Nevertheless, the biggest disadvantage is the potential for contamination of the reaction due to multiple handling of amplicon products and therefore the increased risk of false positives [68].

e) Immunohistochemical Staining and Special Stains

Immunohistochemistry identifies antigens or proteins within cells of a tissue section, then exposes this tissue to the corresponding antibodies of the targeted protein (antigen) where the antibody binds to the antigen. Leptospiral antibodies are not commercially available, and are typically based on LPS or LipL32, which results in poor specificity and abundant background staining. Similar to culture or FAT, immunochemical staining techniques require a certain number of organisms present in the examined tissue. That means they are less sensitive when only low numbers or just localised organisms may be present, as can be the case for very acute infections. On the other hand, these techniques can be useful in cases where the material is not suitable for culture or where a quick diagnosis is needed [6, 15]. Common aniline dyes for bacteria, like Gram stains, do not work on *Leptospira*,

but Giemsa and silver staining like Levaditi or Warthin-Starry (WS) do, although both methods are not sensitive or specific and lead to both false-negative and false-positive results [7].

3. Leptospirosis

"Leptospirosis is a transmissible disease of animals and humans caused by infection with the spirochete *Leptospira*." (WOAH 2022)

The WOAH describes and divides leptospirosis into an acute and a chronic form [8]. It recommends considering leptospirosis as acute in cases of acute agalactia in cattle and sheep; icterus and haemoglobinuria particularly in young animals; meningitis; and sudden renal failure or jaundice in dogs. Conversely, chronic leptospirosis should be included in the differentials with abortion, stillbirth, birth of weak or premature offspring; infertility; chronic renal failure or chronic hepatitis in dogs; and periodic ophthalmia/recurrent uveitis in horses.

a) Pathology

The bacterium enters via mucosa or damaged skin and then spreads haematogenously throughout the whole body [7]. As mentioned above most cases of leptospirosis in maintenance hosts show only subclinical signs; and only an increased antibody titre or a mild interstitial nephritis reveal a former infection. In incidental hosts *Leptospira* causes nephritis, hepatitis, endotoxemia, and haemoglobinuria during the bacteraemia phase of an acute or subacute leptospirosis. After overcoming the acute phase, a chronic manifestation may become established. More specifically an acute, clinical infection often results in jaundice, caused by either haemolysin producing bacteria or toxic/ischaemic hepatic injury. Haemolysin can also result in anaemia. Intravascular haemolysis due to the reaction of antibodies with leptospiral products attached to erythrocytes can also cause destruction of erythrocytes. Whereas interstitial lymphocytic to plasmacytic nephritis and tubular degeneration are common findings with all serovars, clinical renal failure in animals is almost exclusively reported in dogs infected with Canicola and other serovars like Icterohaemorrhagiae, Grippotyphosa, and Bratislava. Reproductive disorders include abortion, stillbirth, infertility, and congenital infections in newborns, and are considered the most important manifestation of leptospirosis in ruminants and swine. While adult pigs are often affected by Icterohaemorrhagiae, Grippotyphosa and Bratislava targeting the reproductive organs, young pigs usually develop systemic infections with high mortality caused by Icterohaemorrhagiae. The most common serovars in cattle are Hardjo (Milk-drop syndrome and abortion) and

Pomona (abortion and stillbirths). Both of these serovars also occur in sheep, mainly resulting in subclinical or sporadic diseases. Recurrent uveitis, or periodic ophthalmia is predominantly a problem in horses with Pomona infections but can develop in all animals [7, 57]. Human beings can be infected by serovars of many pathogenic species, for instance *L. interrogans, L. kirschneri, L. borgpetersenii, L. santarosai, L. weilii, L. alexanderi,* and *L. noguchii* [69]. The highest incidence of human leptospirosis is observed in tropical and subtropical regions and the disease shows a variety of clinical signs from a mild self-limiting illness to a severe and potentially fatal disease [70]. The acute and severe form of leptospirosis in humans is known as Weil's disease and it is characterised by jaundice, kidney failure, and haemorrhages including severe leptospiral pulmonary haemorrhage syndrome (LPHS) [69]. The correlation between the infecting *Leptospira* species and severity and clinical picture of the disease remains not yet fully understood [71].

b) Virulence Factors

Bacteria possess specific attributes, i.e. virulence factors that facilitate their replication and distribution within a host. Different strategies exist to avoid or escape the host immune defence. Identification of and knowledge about virulence factors enhance our understanding of pathogenesis and pathology, and additionally may provide targets for drugs and therapies. Overall, the molecular mechanisms on how pathogenic *Leptospira* cause host invasion and tissue damage are still widely unknown, although some components have been identified [1, 72]. *Leptospira* have several adhesion factors, e.g. fibronectin-binding proteins LigA and LigB (leptospiral Ig-like) [73] and its major surface lipoprotein LipL32 that binds the extra cellular matrix (ECM) of host cells by binding both host laminin [74] and collagen and fibronectin [75]. Also the surface lipoprotein Loa22 is able to bind to ECM and is considered important for *Leptospira* virulence [76] and in triggering an inflammatory responses via interactions with Toll-like receptor 2 and downstream signalling [77]. Leptospiral LPS, in contrast to Gram negative bacteria, is significantly less harmful in endotoxin evaluations, although they share strong chemical and immunological similarities. Several *Leptospira* have been identified with haemolysin and as the name implies these are substances (lipids and proteins) that cause lysis of erythrocytes by damaging the cell membrane. A group of six surface proteins (LenABCDEF) binds both the complement regulator factor H and host laminin, but its significance as a virulence factor is yet to be proven.

4. *Leptospira tipperaryensis*

The vast number and variation of serovars of *Leptospira* present challenges to an everlasting classification. Thus, original historical classifications are long outdated and new and more specific phylogenetic analyses reveal new relatedness and new species. As mentioned above more recent studies divide all *Leptospira* into subclades [5], i.e. subclade P1 (pathogen), P2 (intermediates), and S1 and S2 (saprophytes) or pathogenic *Leptospira* into four sub-branches [78].

In 2016, a *Leptospira* sp. initially suspected to be a serovar of *L. alstonii* was reported for the first time in Europe and additionally for the first time in a mammalian species [38]. Until then, *L. alstonii* had only been described in frogs from China [78] and had been cultured from soils and environmental samples in Japan and Malaysia [79, 80]. Three leptospiral isolates were cultured from urinary samples of 18 wild greater white-toothed shrews (*Crocidura russula*) trapped in County Tipperary in Ireland. They were identified by dark-field microscopy, but typing of these isolates was not possible implying a potential new serovar(s). These bacteria expressed LipL32 and did not grow in the presence of 8-azagunaine as classified for other pathogenic *Leptospira*. Further sequence analysis of 16S ribosomal RNA and secY led to the conclusion that these serovars were closely related to *L. alstonii* although the restriction enzyme pattern was different. Consequently, a new serovar was reported, serovar Room22 (GenBank accession numbers CP015217 (chromosome I) and CP015218 (chromosome II) [15]. Even though initially this serovar was considered to assign to *L. alstonii*, later studies identified it as its own new species, i.e. *Leptospira tipperaryensis* sp. *nov*. relating to Tipperary, a county in in the southern region of Ireland where it was detected [5].

5. Shrews

a) Native Pygmy Shrew - Dallóg fhraoigh

The pygmy shrew (*Sorus minutus*) is widely distributed in Europe and is Ireland's smallest mammal, and until recently its only shrew species. It is considered a native Irish shrew species, as its introduction occurred several thousand years ago according to genetic analysis [81, 82]. Their size ranges between 4.5 and 6 cm with a long slim tail which measures up to 5 cm in length (Fig. 2). Adults weigh approximately 4-6 g. The main morphological feature that distinguishes them from other shrews and small rodents are their teeth; pygmy shrews possess red-tipped teeth due to an iron deposit, which helps provide resistance to

wear. Their territory includes hedgerows, grasslands, woodlands, and peatlands and outside of mating season pygmy shrews are solitary creatures. Females produce up to three times per breeding season and litter sizes are about 4-6 young with a birthweight of about 0.25 g. Their very small size results in an extremely high metabolic rate, which means pygmy shrews need a food intake of 1.25 times of their own bodyweight. Food deprivation of two hours may result in life-threatening negative energy balance. When hunting they mostly rely on their sense of smell and touch, as their sense of sight is very poor thus their Irish name Dallóg fhraoigh (‘blind creature of the heather’). The predominant diet of pygmy shrews consists of beetles, spiders, and insect larvae smaller than 1 cm, as larger pray is too big for a pygmy shrew to ingest. Pygmy shrews are listed as ´least concern´ by the International Union for Conservation of Nature, but the recent introduction of GWTS (*Crocidura russula*) into Ireland has already resulted in local decreases in population numbers and even their local extinction in some regions of the country [83].

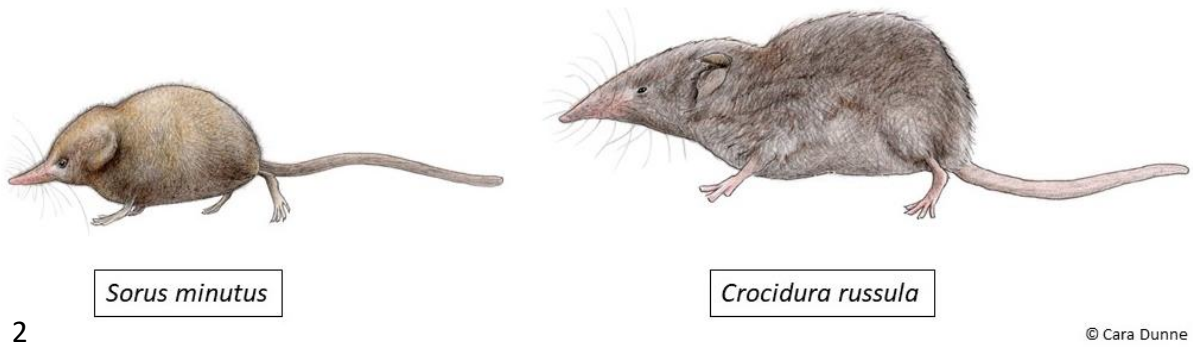

Figure 2. Left: The smaller Irish native pygmy shrew (*Sorus minutus*). Right: The larger invasive GWTS (*Crocidura russula*).

b) Invasive Greater White-Toothed Shrew - Dallóg mór bhánfhiaclach

The GWTS, or Dallóg mór bhánfhiaclach (‘big white-toothed blind creature’), inhabits large areas of Europe and the northern coasts of Africa, and until 2008 had not been reported on the island of Ireland. Adult GWTS are 6-9 cm long with a 3-4.3 cm long tail and their body weight is about 11-14 g, making them about three times bigger than their smaller relative the pygmy shrew (Fig. 2). Similar to the pygmy shrew their typical habitat covers grasslands, woodlands, hedgerows, and agricultural areas. As they have a larger body size and weight compared to pygmy shrews, they feed additionally on small rodents, lizards and small amphibia. GWTS live semi-social, meaning during winter they share a nest and mating pairs defend a common territory. Interestingly the GWTS undergoes only one breeding season per

lifetime with about four litters and up to ten young per litter. Their conservation status is classified as 'least concern'.

c) Conflict between GWTS and Pygmy Shrews in a Shared Habitat in Ireland

A newly introduced species can be of benefit to an ecosystem, e.g. the GWTS is an additional item for birds of prey thus decreasing the pressure on other small mammals like the pygmy shrew [84]. However, shared diet and landscape can lead to direct competition for resources. In western parts of Europe, they are sympatric and the population size of pygmy shrews ranges from uncommon in shared habitats to more numerous in other places (e.g. Belle Île) [82, 85]. In 2014 McDevitt et al published the results of an eight year long study (between 2006-2013) investigating the dynamic of these two species in their new coexistence on the island of Ireland [37]. An intensive analysis was performed on data from live-trapping, sightings, and bird of prey pellets/nest inspections to model the dynamic of the distribution of these two shrew species. They revealed an expansion rate for the GWTS of 5.5 km/year, which is a huge amount for a small mammal. Moreover, they found that the presence of pygmy shrews was negatively associated with the presence of GWTS, i.e. in areas where GWTS are already established pygmy shrews are absent and only found at the very margins of these territories. The pace of this development led to the conclusion that the pygmy shrew did not have enough time to adapt to its new neighbour, and tight monitoring of this development is needed to determine if the decline of the pygmy shrew population continues with the dissemination of GWTS.

d) Leptospirosis in Shrews

There are only a few studies reporting on *Leptospira* spp. in shrew species. One study in Germany detected DNA of the pathogenic *L. kirschneri* in the kidneys of 20% of their examined *Crocidura* spp. (i.e. GWTS) and 6% of the *Sorex* spp. (only in crowned shrews [*Sorex coronatus*], not in pygmy shrews) [33]. Other studies detected DNA of *L. kirschneri* and *L. borgpetersenii* in common shrews (*Sorex araneus*) in Germany [34], *L. kirschneri* in African shrews (*Crocidura cf. olivieri*) in Benin [36], *Leptospira interrogans* (via serology) in the lesser red musk shrew (*Crocidura hirta*) in Tanzania [32] and various serovars (i.e. Australis, Icterohaemorrhagiae, Grippotyphosa, Pyrogenes and Javanica) in red-toothed shrews (*Soricinae*, not further specified) in Siberia [35]. Other studies did not detect *Leptospira* spp. in shrews at all, for example in Afghanistan [86] or Lithuania [31]. None of those studies have

investigated the potential pathological effects of *Leptospira* spp. in shrews, as small mammals like rodents and shrews are known to be maintenance hosts. Having a newly introduced pathogen into the Irish pygmy shrew population would be a great opportunity to examine the pathogenicity of *Leptospira* in a non-adapted host.

6. Dogs

a) Serovars

As mentioned above dogs in Europe are mainly seropositive, i.e. are infected with the serovars Icterohaemorrhagiae, Grippotyphosa, Australis, Sejroe, and Canicola [39]. Serovars that are most likely to cause clinical signs in dogs are Icterohaemorrhagiae, Grippotyphosa, Canicola, Pomona, and Bratislava [87]. Analysis of additional risk factors indicate that male dogs, puppies, herding or working dogs, dogs kept in poor sanitary conditions and dogs with access to outdoor water sources are reportedly at an increased risk for infection with *Leptospira* [88].

b) Clinical Signs

The clinical picture depends (amongst other factors) on the serovar, its virulence, the targeted organ, and the host condition. Clinical manifestation of the disease can be peracute, acute and subacute/chronic. Major organ systems that are targeted by *Leptospira* are the kidneys and liver, but many other organs can be affected: lungs, spleen, vascular endothelia, uvea/retina, skeletal and heart muscles, meninges, pancreas, and the genital tract [88]. In general, Icterohaemorrhagiae tends to predominantly affect the liver, while Canicola primarily causes renal damage. Nevertheless, there is overlap in their clinical manifestation.

In peracute infections, puppies develop fever and haemolytic signs, and death is observed within hours to two to three days after infection. Haemorrhages are also a feature of acute infections, and bacteriaemia causes fever, jaundice, haemolytic anaemia, haemoglobinuria, pulmonary congestion, or occasionally meningitis [7]. Fatal acute infection is often accompanied by hepatic dissociation. After overcoming the acute septicaemic phase, lesions switch from the liver to the kidneys and are mostly interstitial, but also occasional glomerular inflammation and degeneration can be observed. Interestingly, renal failure is almost exclusively described in dogs. Death can follow quickly after developing renal failure, however even subclinical chronic infections, persisting over years, will eventually lead to chronic interstitial nephritis and subsequent renal failure [7].

A somewhat underestimated form of clinical leptospirosis in dogs is the LPHS. This manifestation is potentially life-threatening and has emerged in more recent years in Europe [88-90]. The pathogenic dynamic behind this syndrome is not yet fully understood, but it is likely multifactorial. One important hypothesised mechanism is direct damage to host endothelial cells by pathogenic *Leptospira* leading to increased alveolar permeability [88]. Alternative or additional factors include altered sodium transporter and immunoglobulins, and complement factor deposition [88].

c) Histopathology Findings

In dogs with fatal peracute leptospirosis, dissociation of hepatocytes is a very characteristic but not a pathognomonic histopathological change. Signs of degeneration, i.e. cellular swelling, eosinophilic and granular cytoplasm, karyolysis, karyorrhexis, and nuclear debris as well as signs of regeneration, i.e. cytomegaly, binucleation, and mitotic figures, can be observed.

In the acute phase, the renal changes are characterised by tubular injury rather than inflammatory changes. These changes are mainly found in the proximal convoluted tubules and include epithelial swelling and necrosis. After several days an interstitial oedema develops and diffuse mild infiltration with lymphocytes and plasma cells occurs. With chronicity, unspecified changes like interstitial fibrosis, thickening of Bowman's capsules, and periglomerular fibrosis will develop [57].

d) Treatment and Prevention

Effective treatment of canine leptospirosis involves a combination of antimicrobial therapy and supportive care targeting affected organs. The effect of antibiotics in humans on disease progression is not unanimously proven [91], and even less data is available for canine leptospirosis. However, the European consensus statement strongly recommends the use of antibiotics, specifically doxycycline or intravenous penicillin derivatives for canine leptospirosis, along with therapeutic recommendations for acute kidney injury and *Leptospira*-induced hepatopathy [88]. Treatment includes fluid therapy, correction of electrolyte and acid-base disorders, managing systemic hypertension and gastrointestinal complications, pain management, and nutritional support. For dogs with LPHS, oxygen therapy and mechanical ventilation may be necessary, and plasma transfusions are the main

therapeutic option for dogs with disseminated intravascular coagulation associated with leptospirosis.

Vaccination is the primary method for preventing leptospirosis. Early bivalent vaccination provided significant protection against serovars Icterohaemorrhagiae and Canicola, reducing infection with these strains. However, bivalent vaccines did not offer sufficient cross-protection against other serovars, which led to increased prevalence of serovars from different serogroups, such as Grippotyphosa and Australis [39, 88]. As a response, vaccination schemes have been updated, and current European vaccines now cover four serogroups (Icterohaemorrhagiae, Canicola, Grippotyphosa, and Australis). These vaccines have been shown to offer protection for at least 12 months [92]. The duration of immunity after natural infection in dogs is unclear but is likely to be similar to that induced by vaccination. Considering that dogs can be exposed to infection with serovars from other serogroups, it is recommended to vaccinate them as soon as possible after clinical recovery to ensure continued protection.

Chapter 2

Exploring the Impact of *Leptospira* sp. on Shrews in Ireland

Introduction

In 2016, a new pathogenic *Leptospira* sp., *L. tipperaryensis*, was isolated in Ireland from GWTS, an invasive species, that populates the south of Ireland [5, 15, 38]. This initial study suggested a considerable prevalence (17%, n = 3) of the new bacterial species; however only a small number of GWTS were examined (n = 18). Nevertheless, the role of *L. tipperaryensis* within the Irish ecosystem and its impact on the GWTS and related species is currently unknown.

Leptospira are Gram-negative bacteria of the order spirochaete and the causative agent of leptospirosis, often reported as the most common zoonotic disease [1]. Leptospirosis occurs worldwide and affects a multitude of domestic and wild animal species. Based on outer membrane components, i.e. LPS, *Leptospira* are grouped into serovars which can be divided into pathogenic, saprophytic, or intermediate serovars. To date there are more than 300 serovars of 65 different *Leptospira* species, and new species and serovars are being continuously discovered [5, 43, 44]. Depending on the species affected and the causing serovar, the clinical disease can range from mild fever to severe internal bleeding, jaundice, and kidney failure [1, 53]. More severe signs and clinical disease are usually seen in "incidental" hosts which develop acute leptospirosis with renal pathology characterised by tubular damage and neutrophilic to lymphocytic and plasma cellular interstitial infiltrates. In contrast, "maintenance" hosts infected by specifically adapted serovars present mainly subclinical even if they develop chronic interstitial nephritis [27, 93]. In those carrier animals pathogenic leptospires persist in the epithelium of the proximal renal tubules (located in the cortex), where they display a unique apical distribution (Fig. 1A, B) [1, 94, 95].

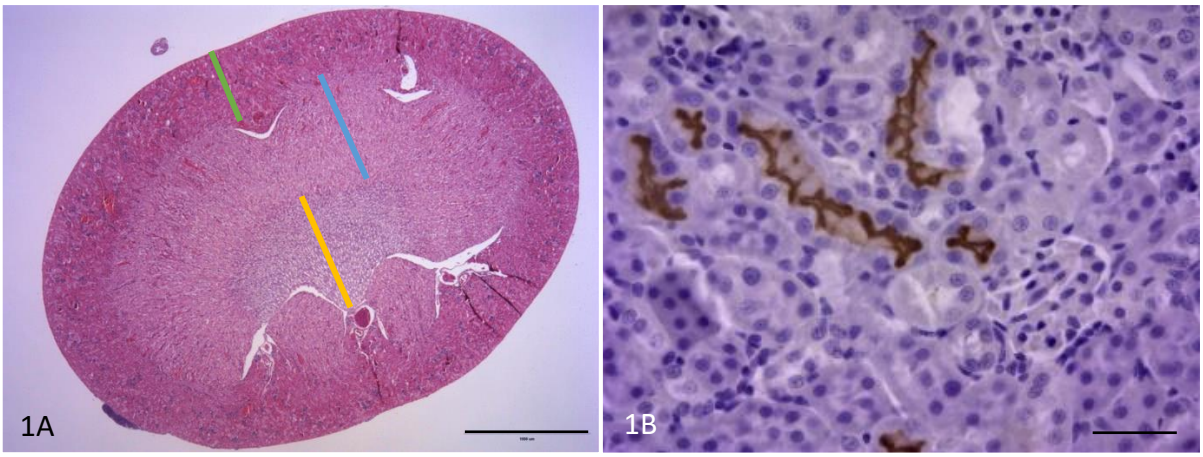

Figure 1. (A) The kidney of most mammals, including shrews, is organised by cortex (green line), medulla (blue line), and pelvis (yellow line). Proximal convoluted tubules

are located in the cortex. Bar 1000 µm. Haematoxylin and eosin stain. (B) Immunohistopathology of kidney from a GWTS in France (control). Leptospires, seen as brown material along the apical border of proximal renal tubular epithelium labelled with LipL32 antibody. Bar 50 µm. IHC.

From the cortex infectious leptospires are intermittently shed in the urine into the environment. They are then transmitted to susceptible animals by direct or indirect contact of damaged skin or mucous membranes with water or soil contaminated by urine [58]. Important reservoirs are rodents, especially rats (mainly *Rattus norvegicus* and *R. rattus*) and other small mammals like mice, voles, shrews, and hedgehogs [1, 30, 33, 53, 86]. For the last 50 years no data on the incidence of *Leptospira* in Irish wildlife has been published [14] except the recent discovery of the new species *L. tipperaryensis* [15].

Ireland's smallest mammal is the pygmy shrew (6 cm and 6 g) which is widely distributed in Europe and is the only native Irish shrew species [81, 82]. In the early 2000s another shrew species, the GWTS (9 cm and 14 g), was identified as a new invasive species in Ireland [96].

Figure 2. Left: The smaller Irish native pygmy shrew (*Sorus minutus*). Right: The larger invasive GWTS (*Crocidura russula*).

Both shrew species share a similar habitat and diet and in many European areas they coexist, especially where pygmy shrews are abundant like on the French island of Belle Île for example [85]. However, recent research has shown that in Ireland the distribution of pygmy shrews is negatively impacted by the presence of GWTS. Pygmy shrews have even been replaced in areas where the latter are fully established, and GWTS are expanding at a high rate [37, 85].

In 2013, a new pathogenic *Leptospira* sp., initially termed *L. alstonii* serovar Room22 and later assigned as *L. tipperaryensis*, was cultured from three urinary samples of 18 trapped wild GWTS [5, 15, 38]. Like other pathogenic *Leptospira* species *L. tipperaryensis* expresses

outer membrane lipoprotein LipL32 and does not grow in the presence of 8-azagunaine [38]. The initial findings suggested that the new *Leptospira* species is prevalent within the newly introduced GWTS population. However, it remains unknown if this new isolate had an impact on the health of GWTS, i.e. whether the GWTS functions as a maintenance or incidental host. Furthermore, the potential spread of the bacteria to the pygmy shrew population of Ireland and its role in the decrease of the native population has not been investigated.

This cross sectional study investigates the prevalence of *L. tipperaryensis* in the GWTS in Ireland, and a possible spread of the infection to the native species. The impact of this new infectious agent on the health of the two shrew species in Ireland is established (maintenance or incidental host) as is its possible role in the decimation of pygmy shrews in the areas of cohabitation. For this purpose, renal tissues of pygmy shrews and GWTS are examined histologically for lesions and subjected to IHC and qPCR to detect the presence of any pathogenic *Leptospira* sp.

Material and Methods

Sample Collection and Sample Size

Pygmy shrews and GWTS were collected by Dr Allan McDevitt for his project 'Modelling the evolutionary consequences of a recent invader in Ireland' [37, 85] in the years 2017 and 2018. Sampling sites in Ireland included the South of Ireland around County Tipperary (Fig. 3) where both species were present together in the 2012/2013 survey [37]. This area covered habitats where pygmy shrews were no longer present (i.e. replaced by GWTS), habitats where both species overlapped, and adjacent areas where GWTS had not yet invaded and where only pygmy shrews were present.

As controls pygmy and GWTS were sampled across the French island Belle Île (Fig. 3) (84 km^2) where both species cohabit.

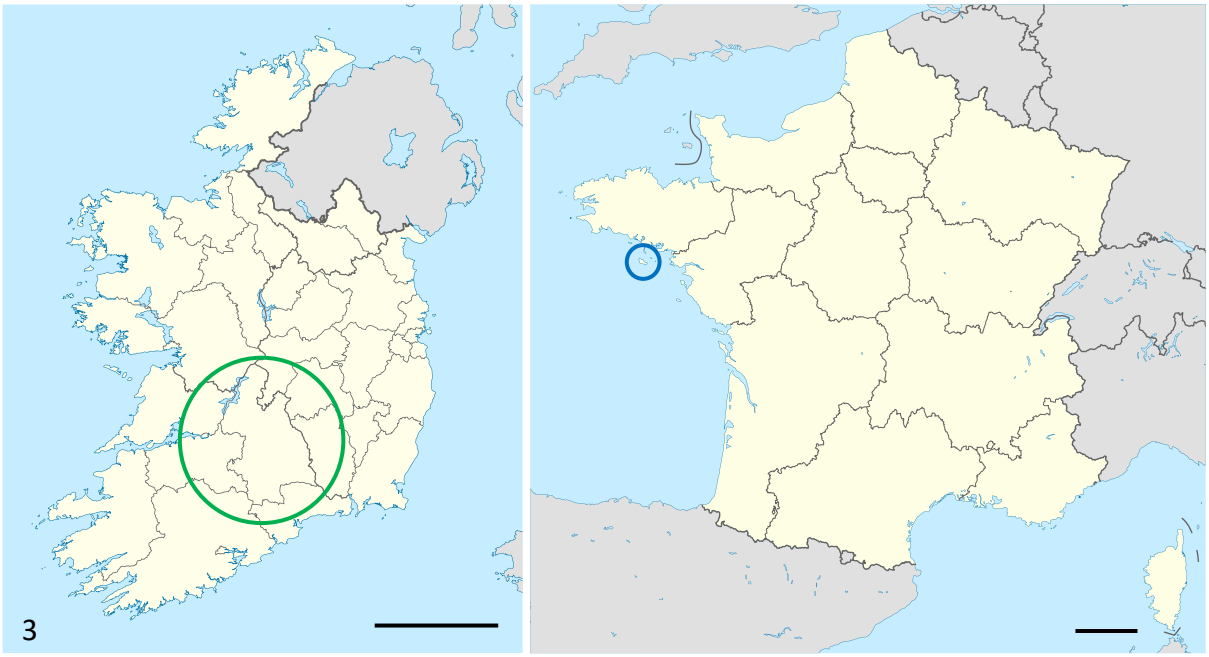

Figure 3. Left: Sample sites in Ireland are centred around the county Tipperary, highlighted in a green circle. Bar 100 km. Image modified and licenced under CC BY-SA 3.0 [97]. Right: Sample site in France is the island of Belle Île, highlighted by a blue circle. Bar 100 km. Image modified and licenced under CC BY-SA 4.0 [98].

All animals were live-trapped using trip-traps (Proctor Bros. Ltd., UK) with no bait. Shrews were immediately euthanised by cervical dislocation following standardised guidelines [6]. All trapping and procedures were performed under the appropriate licences C21/2017 (National Parks and Wildlife Service, Ireland), AE18982/I323 (Health Products Regulatory Authority; Ireland) and A-75-1977 (Belle Île, France), and ethical approvals ST1617-55 (University of Salford, UK) and AREC-17-14 (University College Dublin, Ireland).

A total of 96 pygmy shrews and 134 GWTS were caught in Ireland and a total of 40 GWTS and 41 pygmy shrews were caught in Belle Île. For the purpose of this study, one kidney was placed in 10% buffered formalin. The other kidney was frozen at -80 °C for shrews captured in Ireland only, 74 pygmy shrews and 94 GWTS. The population size of both pygmy shrews and GWTS in Ireland is unknown [99]. Population estimations of the predominant shrew species in the UK (the common shrews, *Sorex Araneus*) which has a comparative habitat, calculate with densities of around 50 per hectare in woodlands and around 20 per hectare in grasslands and other habitats. This results in a total estimated population of shrews in Britain at 41,700,000 (on 24.36 million ha) [100]. If we transfer those numbers to the area of Ireland (8.44 million ha), we assume to have approximately 14,447,190 shrews in Ireland. With our sample size of 230 shrews sampled in Ireland, we fulfil a 95% confidence level with a confidence interval of ± 5.48% to assess the prevalence of *Leptospira* infection in shrews in Ireland. For comparison, if we assume an unknown (infinite) population the confidence interval would be in a very similar range of ± 5.37 %.

Confidence interval:

Unlimited population:

$$CI = \hat{p} \pm z \times \sqrt{\frac{p(1-p)}{n}}$$

Finite population:

$$CI' = \hat{p} \pm z \times \sqrt{\frac{\hat{p}(1-\hat{p})}{n'} \times \frac{N-n'}{N-1}}$$

z is the z score

p̂ is the population proportion

n and n' are the sample size

N is the population size

Sample size:

Unlimited population:

$$n = \frac{z^2 \times \hat{p}(1-\hat{p})}{\varepsilon^2}$$

Finite population:

$$n' = \frac{}{1+\frac{z^2 \times \hat{p}(1-\hat{p})}{\varepsilon^2 N}}$$

z is the z score

ε is the margin of error

N is the population size

p̂ is the population proportion

Histopathology and Grading

In total we evaluated renal tissue from 311 shrews, which were composed of 18 GWTS from the initial shrew study carried out in Ireland from 2012/2013 [38], and in 2017/2018 collected further 212 shrews in Ireland (96 pygmy shrews and 116 GWTS) and 81 in France (41 pygmy shrews and 40 GWTS), respectively.

For histopathological examination tissues were fixed in 10% buffered formalin, processed routinely, sectioned at 5-µm-thick and stained with haematoxylin and eosin (HE)

according to standard protocols. Histopathological findings were described and interpreted for their potential association with leptospirosis. Typical findings for acute leptospirosis include prominent tubular damage with neutrophilic infiltrates, that later change to lymphocytic, plasmacytic and histiocytic infiltrations. Chronic leptospirosis is characterised by chronic tubulointerstitial nephritis, with infiltrations of lymphocytes and macrophages, variable amounts of fibrosis and subcapsular scarring [27].

Interstitial nephritis was semi-quantitatively graded as mild, moderate, or severe (Fig. 3). Mild inflammation was defined as low numbers of inflammatory cells affecting < 5 % of the tissue section or single (focal) areas. Moderate inflammation was characterised by medium numbers of inflammatory cells affecting 15-20 % of the tissue section or multifocal areas. Severe inflammation was characterised by high numbers of inflammatory cells affecting multifocally > 15-20 % of the tissue section.

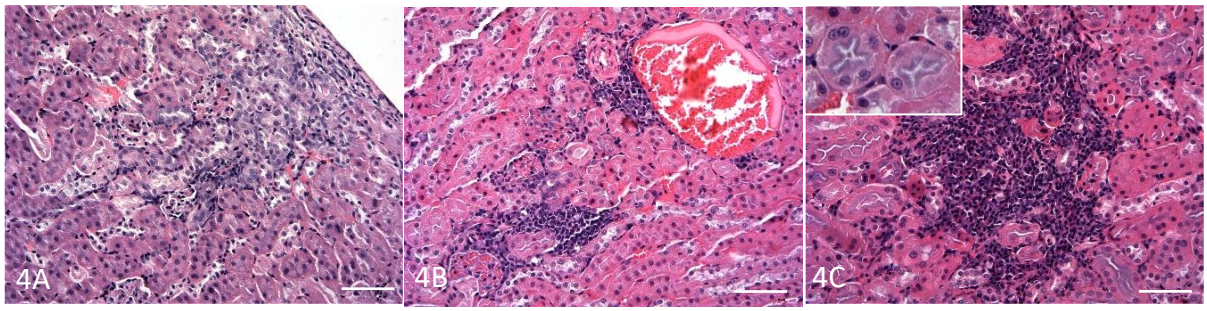

Figure 4. Kidney, grading of interstitial nephritis. (A) Mild interstitial nephritis, low numbers of neutrophils and lymphocytes are scattered between tubules. Bar 100 µm. Haematoxylin and eosin stain (HE). (B) Moderate interstitial nephritis, medium numbers of lymphocytes, plasma cells, and macrophages are separating tubules. Bar 100 µm. HE. (C) Severe interstitial nephritis, high numbers of lymphocytes, plasma cells, and macrophages are separating and effacing tubules. Note the prominent presentation of leptospiral bacteria, visible as a blurred basophilic apical layer on epithelial cells. Inset: Magnification of prominent leptospiral bacteria. Bar 100 µm. HE.

Immunohistochemistry

Renal tissue of pygmy shrews and GWTS was tested with an antibody raised in rabbits against LipL32 (kindly provided by Dr. Jarlath Nally, USDA Agriculture Research Service, Aimes, Iowa, USA), a major outer membrane protein present on all pathogenic *Leptospira* and a rabbit antibody raised against the LPS of *L. tipperaryensis*, designated anti-room22, (kindly provided by Colm Gilmore, Leptospirosis OIE Reference Laboratory, Agri-Food and Biosciences Institute, Veterinary Sciences Division, Stormont, Belfast, Northern Ireland), the recently discovered new *Leptospira* species in GWTS in Ireland [5, 15, 38].

To perform IHC, we applied standard protocols. Briefly, 5-µm sections of formalin fixed and paraffin embedded kidneys were cut and placed on positively charged glass slides (Bond Plus slides, Leica Microsystems Ltd, UK). Paraffin was removed from sections with xylene and ethanol and slides were subsequently rehydrated using Leica autostainer© (Leica Microsystems Ltd, UK). For antigen retrieval we placed the slides in 0.01 M sodium citrate buffer (DakoCytomation Target Retrieval Solution Citrate pH 6 (10x), S2369) and heat-treated them for 10 minutes in a pressure cooker (Pascal DakoCytomation) before cooling them down in PBS (Phosphate buffered saline, Sigma-Aldrich, United Kingdom) at room temperature (RT). Nonspecific staining of tissue sections was minimised by using 10 % (ready to use) horse serum (Vector Labs, USA) with incubation at RT for 30 min prior to incubation overnight at 4 °C with primary antibody anti-LipL32 or anti-room22 at a dilution of 1:1250 or 1:500 respectively. Unbound primary antibodies were removed with a PBS washing step. To block endogenous peroxide activity, we used a 1% H_2O_2/ methanol mixture. As secondary kit, tissue sections were incubated with anti-rabbit immunoglobulin (Vector Labs, USA) for 60 min at RT. After being washed, DAB (3,3'-Diaminobenzidine, Sigma-Aldrich, United Kingdom) was used as chromogen for 10 minutes at RT and all slides were counterstained with haematoxylin before dehydration in alcohols and propane (xylene substitute), and coverslips were then mounted.

For positive controls we used the renal tissue of an experimentally infected guinea pig and the kidney of a dog with confirmed leptospirosis and moderate antigen content in the kidney. Negative controls included "omit" primary, secondary, both antibodies and a goat immunoglobulin G rabbit isotype control (Thermo Fisher Scientific), to rule out any non-specific staining.

Optimisation of Anti-Room22 Immunohistochemistry

To identify *L. tipperaryensis*-positive GWTS from the initial study of 2013 [38] we used a variation of different parameters. In detail, we increased antigen retrieval with the pressure cooker from 5 min to 10 min to 20 min. Primary antibody concentrations were increased from 1:1250 to 1:500 to 1:250 and secondary antibody was increased from 1:1000 to 1:500 to 1:250. Due to lack of specific control tissue for *L. tipperaryensis* cases and failure to create a positive signalling in those original samples, we discontinued the use of anti-room22 antibody. In addition, the samples obtained from the study carried out in France showed

strong immunopositivity (carrier pattern) with the anti-room22 antibody, i.e there was cross-reactivity of anti-room22 with pathogenic *Leptospira* species different from *L. tipperaryensis.*

PCR Extraction

Two protocols were applied to extract DNA from formalin-fixed paraffin-embedded (FFPE) and freshly frozen material respectively. From a total of 13 samples (three samples from the study carried out in France and 10 from the 2013 study carried out in Ireland) FFPE material of 5-10 µg thick sections (not more than 25 mg) of the tissue block containing the kidney were prepared and collected in a tube. Using the QIAamp® DNA FFPE Tissue Kit (Qiagen) according to the manufacturer's instruction we mixed and vortexed the tissue with 320 µl deparaffinization solution (Qiagen). After 3 min incubation at 56 °C 180 µl lysis buffer ATL was added. Centrifugation for 1 min at 10,000 RPM separated the mixture and 20 µl Proteinase K (Qiagen) added to the lower phase initiated the lysis process. Incubation for an hour each at 56 °C and 90 °C was followed by transferring the lysed section into a new tube and the sample was treated according to the steps in the manufacturer's instruction. Finally, the nucleic acid was diluted in 75 µl elution buffer (ATE).

Secondly, to extract DNA from fresh frozen tissue (kidneys) from the 168 shrews sampled in Ireland (74 pygmy shrews, 94 GWTS) we used QIAmp DNA Mini Kit (Qiagen) as recommended by the manufacturer with modifications. Briefly, 25 mg of tissue was dissected with a sterilised scalpel and placed in a 2.0 ml SC Micro Tube PCR-PT (Sarstedt) together with a clean stainless-steel bead (Qiagen). Two minutes at maximum speed on a Qiagen Tissue Lyser homogenised the sample and subsequently we added 20 µl Proteinase K for further lysis. From there we followed the manufacturer's protocol and ultimately nucleic acids were eluted in 200 µl elution buffer (AE).

DNA Quantification and Dilution

After both protocols, microvolume spectrophotometry (NanoDrop™ Thermo Scientific™) facilitated measuring the concentration of nucleic acid within the samples. For optimal qPCR conditions, all samples were diluted with DNase free water to a final concentration of 25 ng/µl for a total amount of 125 ng in 5 µl sample volume.

qPCR

The qPCR on fresh and fixed tissue was carried out using two primer pairs. To detect LipL32, an outer membrane protein that is expressed by all pathogenic *Leptospira* species, we

used 0.2 µl of forward primer LipgrF2 (5'-CGCTGAAATGGGAGTTCGTATGATTTCC-3') and 1.2 µl of reverse primer LipgrR2 (5'-GGCATTGATTTTTCTTCYGGGGTWGCC-3') (Eurofins Genomics UK Limited) [101, 102]. To target *L. tipperaryensis* specifically we designed primers that targeted an equivalent sequence as for the LipL32 primers which enabled us to use the same probe and we used 0.6 µl of forward primer Ltipp-FOR (5'-GGTTCCTGCTGTTATCGCTG- 3') and 0.6 µl of reverse primer Ltipp-REV (5'-CTTCTGGAGTTGCAGCTTTGA-3') (Eurofins Genomics UK Limited). Both primer pairs were of a 100 µM solution and used the probe LipgrP2 (5'-AGGCGAAATCGGKGARCCAGGCGAYGG-3') (Eurofins Genomics UK Limited) with a volume of 0.4 µl and 0.6 µl respectively. The FastStart Universal Probe Master (Rox) (Roche, USA) containing TaqMan DNA polymerase, reaction buffer, nucleotides (dATP, dCTP, dGTP, dUTP), and a reference dye was used according to the manufacturer's protocol and using 5 µl of DNA template in 15 µl reaction (primer, probe, FastStart mix, water). A 7500 Fast Real-Time PCR System (Applied Biosystems, software version 2.0.6) was used with MicroAmp Fast optical 96-well plates and Optical Adhesive Films (Applied Biosystems). Amplification was performed using 20 µl/well and the following thermal cycle: 95 °C 10 min, and 40 cycles: 95 °C for 15 s, 60 °C for 1 min. For technical replication we performed qPCR in duplicates and results were corrected for amplification efficiency. Controls comprised negative controls without template using water and dilution buffer, and positive controls from previous confirmed diagnostic samples and extracted DNA from cultured *L. tipperaryensis* and *L. interrogans*. A standard curve was generated using five-fold serial dilutions for each gene of interest (*LipL32*, *Ltipp*) as well as for reference genes (GWTS, Eukaryotes). Dissociation curves were examined for each gene to ensure specificity of amplification.

Statistical Analysis

Statistical analysis was performed with Graph Pad Prism® 9 software. Simple or multiple t-tests were applied using a normal distribution of data. Differences were regarded as significant at a level of $p \leq 0.0001$ (****).

Results

Immunohistochemistry

Using LipL32 antibody for IHC successfully identified 28 shrews positive for *Leptospira* species antigen. All of those belonged to the shrews sampled in France which resulted in a prevalence of 33.3% (27/81). No carriers were identified in the shrews sampled in Ireland, which was statistical significant (Fig. 5A). Significantly more positive cases were observed in GWTS (23 GWTS, 4 pygmy shrews; Fig. 5B).

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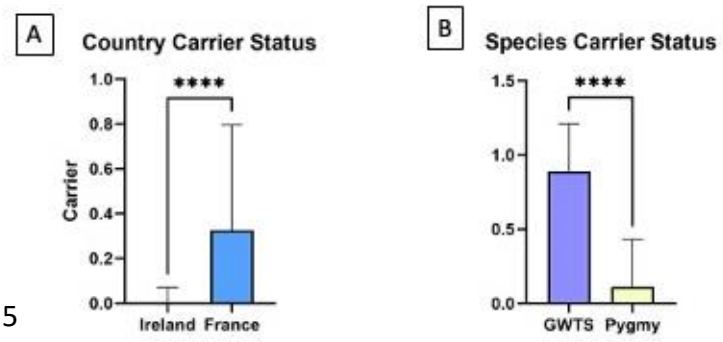

Figure 5. (A) Significantly more French shrews were carriers compared to Irish shrews. (B) Significantly more greater white-toothed shrews (GWTS) were carriers compared to pygmy shrews.

The positive cases were designated carriers of *Leptospira* as these displayed a typical accumulation of brown labelled spirochaetes in the apical border of the proximal tubular epithelial cells, as depicted below (Fig. 6A, B).

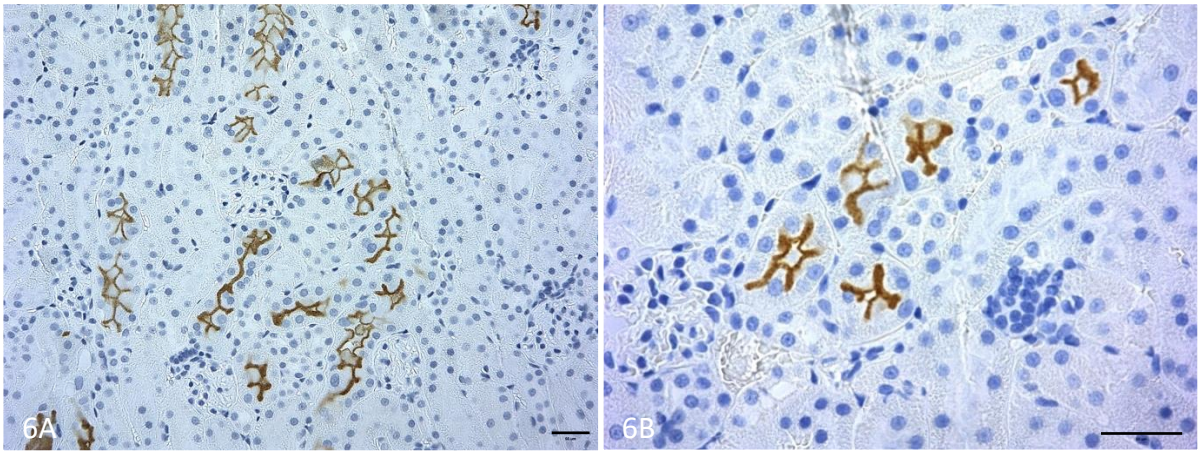

Figure 6. Kidney, proximal tubules. Brown positive labelling is present on apical border of tubular epithelial cells (A), with higher magnification in (B). Bar 50 μm. IHC LipL32.

Animals that did not show the above carrier signalling were categorised as “non-carriers”. Interestingly, six of the GWTS from the study carried out in Ireland displayed multifocal areas of granular, brown labelling in the proximal tubular epithelial cells which differed from the well-defined apical labelling of carriers (Fig. 7). In addition, only one of those animals showed significant moderate inflammatory changes (for details see below). These cases were classified as inconclusive.

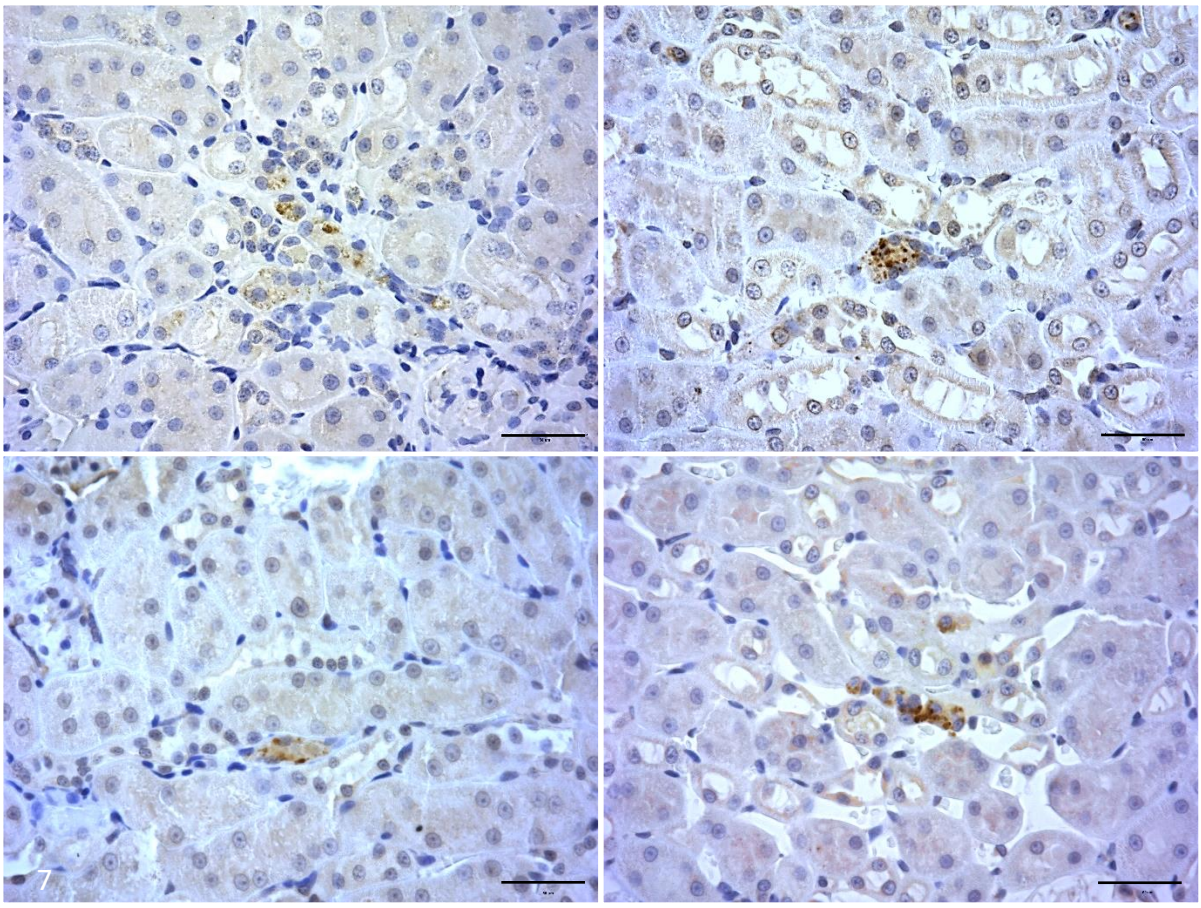

Figure 7. Kidney, proximal tubules. Brown granules are present within cytoplasm of tubular epithelial cells. Bar 50 µm. IHC LipL32.

DNA Quantification and qPCR

Extraction of FFPE material DNA concentration ranged from 294.4 to 1,226.1 ng/µl (Appendix, Table A1) and the fresh frozen material resulted in a DNA concentration from 34.4 to 433.8 ng/µl (Appendix, Table A2), measured with NanoDrop™ Thermo Scientific™.

The concentration of DNA extracted from FFPE material of carrier animals from the control group yielded positive results for the LipL32 primers, but not for the Ltipp primers, confirming that FFPE material was suitable for qPCR. The subset of FFPE material from the

shrews sampled in Ireland from 2013 did not amplify any product with either of the primer pairs.

The qPCR of the fresh frozen tissue of 168 Irish shrews was negative for both primer pairs. These samples included one of the samples with granular immunohistochemical labelling.

Histopathology Findings and Carrier Status

Chronic interstitial nephritis characterised by varying numbers of multifocal infiltration of lymphocytes, plasma cells and macrophages in the absence of fibrosis was observed in 21% (64/311) of the shrews (Fig. 8). Chronic inflammatory changes in shrews were mostly mild (10%; 30/311) and moderate (9%; 29/311), and less often severe (2%; 5/311).

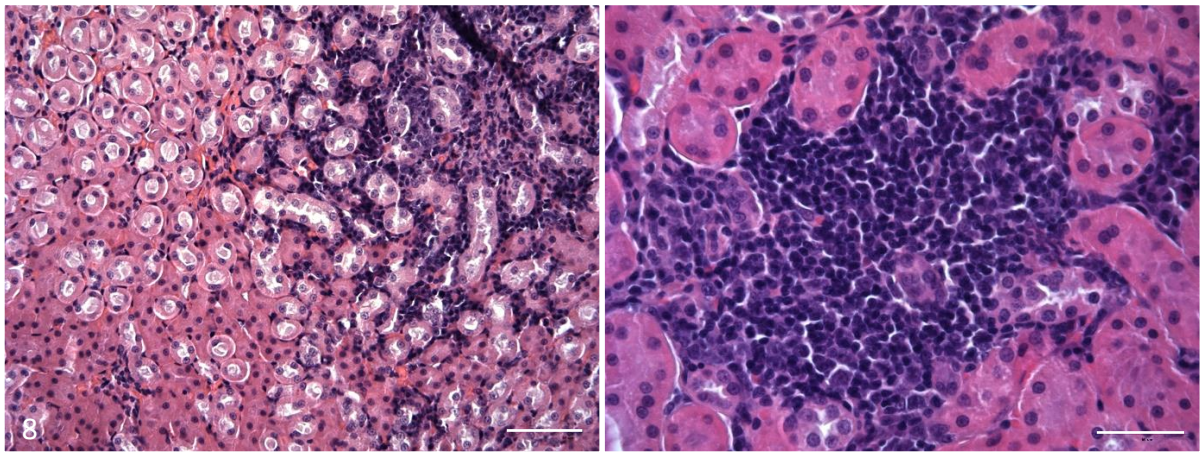

Figure 8. Left: An inflammatory infiltrate expands the interstitium separating tubules. Bar 100 µm. Haematoxylin and eosin stain (HE). Right: The infiltrate is composed of lymphocytes, plasma cells and macrophages. Bar 50 µm. HE.

Findings consistent with acute renal pathology were only observed in 5% (17/311) of shrews. Changes were exclusively present in the renal cortex. Tubular degeneration was characterised by hypereosinophilic attenuated to pale swollen epithelium with karyorrhexis (Fig. 9, left). Tubules were dilated and frequently contained cellular debris. Small to moderate numbers of neutrophils, often with a ring-shaped nucleus (so called ring cells) [103], infiltrated the cortical interstitium, separating and infiltrating tubules (Fig. 9, right). Lesions ranged from focal to multifocal in distribution.

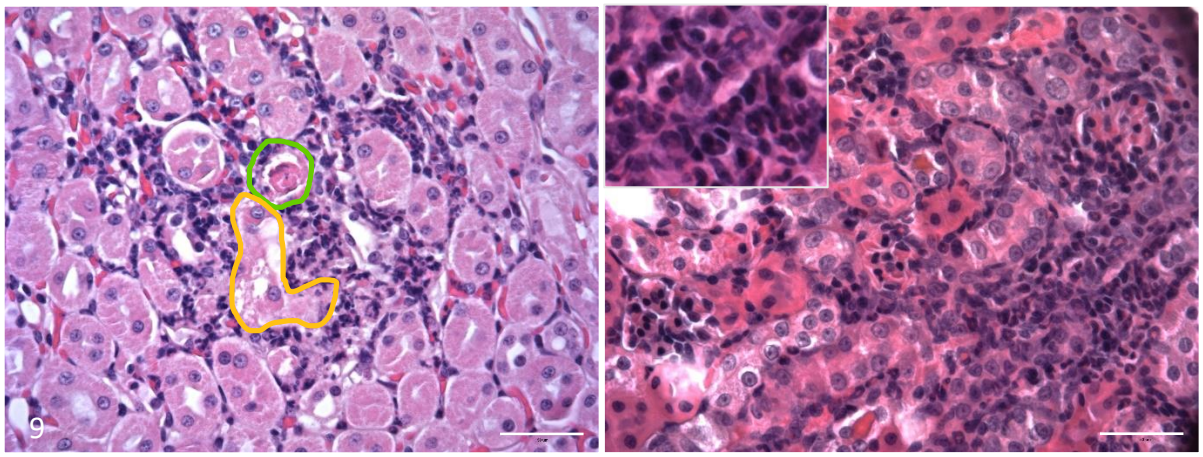

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Figure 9. Left: Tubular epithelium is flattened and hypereosinophilic (degenerated, green mark) or pale and sloughing off (necrosis, yellow mark). Bar 50 µm. Haematoxylin and eosin stain (HE). Right: Tubules are separated by moderate numbers of interstitial neutrophils. Inset: Higher magnification of neutrophils. Bar 50 µm. HE.

Non-specific and less common findings included protein casts, glomerular changes (membranous glomerulopathy), acute haemorrhage, and hydronephrosis. All changes are summarised in Table A3 of the appendix.

The incidence of inflammation was significantly higher in carrier animals compared to non-carrier animals, as shown in Fig. 10A. Additionally, the chronicity of inflammation (Fig. 10B) and the severity of inflammation (Fig. 10C) were significantly greater in carriers.

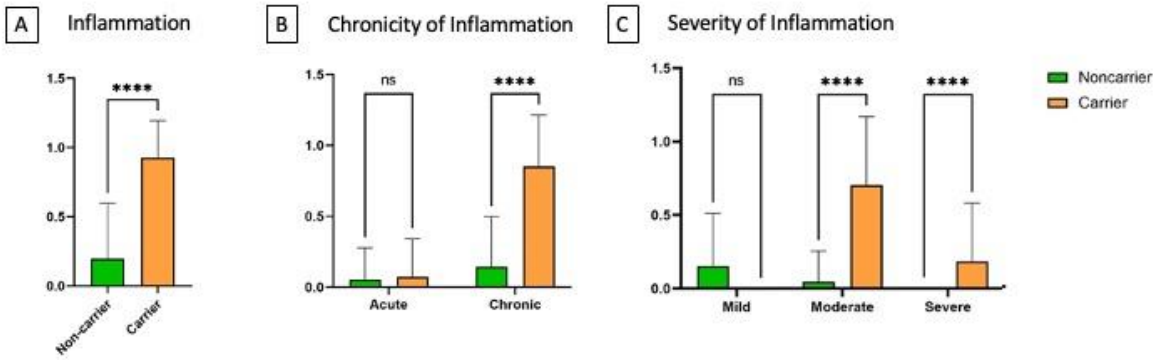

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Figure 10. (A) Carriers presented more frequently with inflammation. (B) Inflammation in carriers was significantly more chronic compared to non-carriers. (C) The severity of inflammation in carriers was significantly higher (moderate and severe).

No statistical difference in the degree of inflammation was observed between the non-carrier animals in those sampled in either Ireland or France. The six inconclusive animals

were all GWTS sample din Ireland, five of them from the initial study collection in 2013. Of those animals one from 2013 had a mild and one shrew from 2018 had a moderate multifocal neutrophilic interstitial nephritis. The other shrews had no significant findings.

Discussion

None of the shrews sampled in Ireland was positive for *Leptospira* sp. in this study, whereas 33.3% of the shrew population of French Belle Île were identified with leptospiral antigen. It is clear from the results of this study that *L. tipperaryensis* or other *Leptospira* spp. do not pose a threat to shrews in Ireland and therefore are not likely to negatively impact their number or distribution.

The absence of infection in this study stands in contrast to the original detection of *L. tipperaryensis*, where *Leptospira* sp. was detected in 17 % (3/18) of GWTS [38]. The original study took place in 2013 whereas our samples were collected in 2017 and 2018. A possible explanation could be that although highly prevalent in the first sampling time frame this (or any other) *Leptospira* species did not manifest in the shrew population from Ireland, but was rather naturally eliminated in the following years. Influencing factors for example could be increased immunity within the population or changes in population density (and thus altered transmission opportunities). Furthermore, the small sample size from the original study might not have reflected the true prevalence of infection within the shrew population, but possibly a localised event. There is no published data in the last 50 years on the incidence of *Leptospira* infection in Irish wildlife [14]. The situation in other European countries is very variable: 35% of brown rats in a French study closely mirror the prevalence of infection found in the shrews sampled in Belle Île. A recent study from the UK detected *Leptospira* in 14 % of rats [104], even though historically prevalences between 50 and 70% are described [104]. Between 6 and 20 % of shrews in Germany [33] and 4.4 % of voles and mice in Lithuania were found to be infected with *Leptospira* sp. [31]. Studies from Lithuania and Afghanistan [31, 86] analysed shrews of similar sample size to our study but did not detect leptospiral DNA in these animals. A possible explanation for these findings might be that shrews are less susceptible to infection with *Leptospira* compared to other small mammals or rodents and this could have contributed to a relatively rapid elimination of *L. tipperaryensis* from the population in Ireland. Furthermore, it appears that leptospirosis in wildlife is less prevalent than previously reported, possibly due to greater rodent control or changes in the population immune status.

Significantly more GWTS than pygmy shrews were detected to be carriers. Both pygmy shrews and GWTS are potential carriers of leptospirosis [33] and the dominance of GWTS carriers is clearly apparent. Similarly, a recent German study detected a much higher

prevalence of *Leptospira* sp. in GWTS compared to other shrew species [105]. There are no studies that explore different susceptibility of various shrew species to leptospiral infection, but potential explanations include differences in immune response or behaviour. More specifically, GWTS are known for their behavioural flexibility to expand their territories and alter ecological niches by exploiting new resources [105, 106]. This may lead to greater exposure to *Leptospira* sp. compared to other small mammals.

Although the population size of pygmy shrews and GWTS in Ireland is unknown [99], if we extrapolate from population densities of shrews in Britain we fulfil a 95% confidence level with a confidence interval of ± 5.48 % and ± 5.37 % for an unknown, infinite population. These are conventional margins of error and indicate that the size of our sample collection was appropriate and our findings representative for the shrew population in Ireland.

The association of the interstitial nephritis and leptospirosis is well known [7, 53, 107] and although carriers are considered to be clinically silent, interstitial nephritis is the predominant lesion in rats and other carriers [26-29]. Our results show that both the presence of nephritis and the degree of inflammation was significantly higher in carrier animals compared to non-carriers, and helps validate the tests we used. No significant differences were observed regarding the degree of inflammation in the non-carrier animals sampled in France and Ireland. This supports the suitability of the control group. Animals that were not categorised as carriers displayed significantly less renal inflammation, and those cases were mostly mild compared to moderate nephritis in carrier animals. These inflammatory changes were not considered to be of clinical significance, but rather a background finding and were equally distributed in shrews sampled in both France and Ireland.

The archival material obtained from the 18 GWTS that in 2013 yielded the three new isolates did not feature any classical positive carriers as defined by IHC. This may suggest that the GWTS is not a maintenance host for *L. tipperaryensis.* Furthermore*,* as the use of *L. tipperaryensis* specific antibodies did not provide any unambiguous labelling, no suitable control was obtained and thus the use of this antibody was discarded. Unfortunately, due to labelling issues it could not be ascertained which of the 18 original cases had been positive for *L. tipperaryensis* by urine culture. Even though IHC provided the most reliable results in this study, the lack of commercial antibody for *Leptospira* and the cross reactivity between serovars (as seen in this study as well) can produce false negative and false positive results and thus impair this detection method. Initial trials on FFPE material of carriers sampled in

France generated amplification of *Leptospira* in qPCR and positive controls included diagnostic cases of leptospirosis. The reported sensitivity of LipL32 conventional PCR for rodent kidney samples is 102 leptospires/ml and the sensitivity of the real-time PCR is assumed to be comparable with three to five copies [101, 108, 109]. While it is unlikely that the samples used in this thesis were below this threshold, it is not impossible. The failure to detect *Leptospira* with one method highlights the sensitivity problems of different tests, and emphasises the importance of using multiple detection methods in parallel [63, 110, 111].

Interestingly, inconclusive (questionably positive) granular labelling by IHC was observed in six cases, five of which were from the original 18 shrews [112, 113]. For two of these cases both IHC antibodies showed similar patterns. Only mild acute interstitial nephritis was seen in two out of these cases, which stands in contrast with other findings in this study where *Leptospira* was frequently associated with more advanced inflammation. Still, those advanced lesions were interpreted as chronic leptospirosis and the inconclusive labelling could be an early, acute infection. In the absence of other test results such as PCR or MAT, which could not be done due to lack of serum and fresh tissue, no definite conclusion can be drawn and further investigation, e.g. MAT if serum was available or fresh tissue qPCR, would be needed.

The findings of this study do not support the suggestion that *Leptospira* sp. play a role in the on-going expansion of the invasive GWTS and simultaneous replacement of the native pygmy shrews. In line with this, new studies rather indicate increased dietary overlap and concurrence on food resources between the larger invasive species and the smaller native species as the main contributor to the observed changes in population dynamics [85].

One limitation of this study was the lack of suitable positive control tissues for the IHC and species-specific antibodies. Fresh tissue from the French control group could have enhanced optimisation of qPCR methods, and thus increased detection rate and provided the opportunity for further sequencing. We were not able to determine the serovars of the infecting leptospires and serological classification by microagglutination, the gold standard method of confirming infection in human patients. This would have required additional tissue, such as urine or blood. In this study IHC provided an effective tool in detecting chronic carriers of *Leptospira*, but uncertainties remain as to the detection of acute clinical infections.

Future studies are needed to optimise the diagnosis of leptospirosis in wildlife. These would ideally be base on the use of multiple tissue samples so that IHC, molecular biological

methods, and serology could be deployed. It would be of interest to screen wild and domestic animals in Ireland for *L. tipperaryensis,* to investigate the origin of this species and whether it is maintained in any other animal species.

Conclusion

We detected no leptospiral infection and only a low prevalence of nephritis in shrews in Ireland and conclude that leptospirosis does not impact the health of either shrew species of shrews on the island, or contribute to the local replacement of native pygmy shrews. Leptospirosis was associated with chronic moderate to severe nephritis predominantly in GWTS from Belle Île, France. A background mild, mainly acute inflammatory change was observed in 5% of shrews not confirmed as infected with *Leptospira* sp.

Although the detection of *Leptospira* sp. in this study was challenging, we successfully identified the presence of the pathogen on FFPE material in carriers using IHC. The use of qPCR to detect *Leptospira* can be further optimised, which is why we highlight the necessity to use multiple diagnostic tests and to target multiple leptospiral genes to optimise the detection rate of this pathogen.

Chapter 3

Investigating Exudative Glomerulonephritis and Leptospirosis in Dogs

Introduction

Exudative GN is a rarely described renal pathology in dogs [114] of unknown aetiopathogenesis. In the last 20 years exudative GN has been frequently diagnosed in dogs in Switzerland that presented clinically as suspect cases of leptospirosis, but where an association with this infection has not been demonstrated.

Leptospirosis occurs worldwide and the distribution of serovars varies between countries and regions. The main disease causing serovars in European dogs are Icterohaemorrhagiae, Grippotyphosa, Australis, Sejroe and Canicola [39], while the serovars Australis and Bratislava show the highest seropositive rates in Switzerland [90]. Dogs are maintenance hosts for *Leptospira interrogans* serovar Canicola, and clinical disease is primarily caused by other serovars of the species: *L. interrogans* and *L. kirschneri*. Vaccination against leptospirosis has markedly reduced the prevalence of the disease in many countries, and particular combinations of serovars occur in particular regions [54, 115, 116] are available.

Severe renal necrosis, hepatic necrosis, and pulmonary haemorrhage are all manifestations of canine leptospirosis [7, 117] and the disease usually occurs in peracute or acute forms in this species. The peracute form is most often observed in puppies infected by Icterohaemorrhagiae, which develop a fulminant septicaemia with fever, haemorrhage and potential death within hours or days. An acute, often severe form of leptospirosis occurs in young animals which can develop a range of clinical signs, e.g. fever, haemorrhage, vomiting and diarrhoea, pneumonia, hepatitis and/or hepatocellular injury, intravascular haemolysis (and icterus), interstitial nephritis and/or renal failure. As a generalisation, infections with Canicola predominate in the kidney, whereas Icterohaemorrhagiae infections manifest in the liver.

Typical light microscopic findings in the kidneys of acutely affected dogs include neutrophilic to lymphoplasmacytic tubulointerstitial nephritis, with or without tubular epithelial injury, i.e. degeneration and necrosis. The damaging effects on glomeruli are largely subtle and unspecific, e.g. mesangial hyperplasia [118], reactive mesangio-proliferative glomerulonephritis [119], or glomerular degenerative changes [53]. However, during the last 20 years dogs in Switzerland with suspected acute leptospirosis have increasingly presented with severe, rarely previously reported glomerular pathology characterised by abundant extravasated erythrocytes and lesser neutrophils accompanied by marked fibrin exudation into the urinary space which has been interpreted as an exudative GN. While an association

between the infection and this unusual pathological change has been suspected, MAT or WS stains have failed to confirm this hypothesis. Identifying a causative agent of this rare pathology is important for the diagnosis, treatment and prevention of future cases.

Diagnosing leptospirosis can be challenging and a multitude of tests are available to either directly demonstrate *Leptospira* in tissues/material, or indirectly by detecting leptospiral antibodies. Serological testing, especially MAT, is the most widely used test for diagnosing leptospirosis and this assay is very useful for detecting acute infection when high antibody titres are present. It can also be used to specify the serovar involved. The use of MAT is limited during very early infection (within the first week), when immunoglobulin concentrations have not yet risen, or in the case of novel serovars (like a regionally uncommon or newly introduced serovar) [6, 111]. Immunohistochemistry may demonstrate leptospiral antigen [112], which is advantageous when diagnostic material is not suitable for culture, for instance when bacteria are fragmented (not viable) or where a rapid diagnosis is required. Unfortunately, antibodies to detect these organisms are not commercially available, and the success of this method can be limited in acute cases, where organisms may occur in low numbers. A variety of assays based on PCR can detect leptospiral nucleic acids and both real-time/quantitative and traditional formats feature high sensitivity and specificity [111, 120]. Their advantages lie in early detection (before antibody titre rise) and that they do not rely on viable intact organisms, unlike culture-based methods. Additional limitations of diagnostic tests include, but are not limited to, amplification inhibitors, and previous antibiotic treatment [53, 111].

This retrospective study was initiated to investigate a possible association of the novel renal findings, i.e. exudative GN, with leptospiral infection. Therefore, FFPE tissues of dogs with suspected/not confirmed leptospirosis and cases with exudative GN available from the archives of the Institute of Veterinary Pathology, Zurich and the Institute of Animal Pathology, Bern, Switzerland were examined for *Leptospira* antigen using IHC and qPCR. Additional available clinical and pathological data was collected from the cases of exudative GN, in order to more clearly define these unusual cases.

Material and Methods

Case Selection

This study included dogs that underwent postmortem examination in the Institute of Veterinary Pathology, Zurich and the Institute of Animal Pathology, Bern, Switzerland during a 20-year period between 1996 to 2015. This institue's archives were searched for cases that either had a suspected or confirmed diagnosis of leptospirosis, or had received a histopathological diagnosis of exudative glomerulonephritis. Exudative GN was defined as abundant extravasated erythrocytes and lesser neutrophils accompanied by marked fibrin exudation into the urinary space. Ninety-nice cases of suspected canine leptospirosis were retrieved from the archives. These diagnoses of leptospirosis were based on the animal's clinical presentation and by an accompanying positive WS stain (n = 49). Fifty of the 99 cases were diagnosed with exudative GN of which 11 were WS positive. Cases that did not present with exudative GN, but showed other typical leptospiral lesions, e.g tubulointerstitial nephritis, tubular necrosis, were termed 'classical' leptospirosis cases. The records did not contain cases of exudative GN that were not suspected to be leptospirosis.

A review of the necropsy reports of those cases followed, and where available we evaluated supporting data. This included signalment, season, geographical location, clinical and laboratory data, and pathological changes in all organs, in particular changes in lungs, liver, and kidneys.

Histology and Warthin-Starry Stain

FFPE renal tissue blocks from the archives were retrieved and HE stained slides examined to describe glomerular changes. Additional renal pathology was recorded, such as tubular necrosis and regeneration, as well as interstitial changes including haemorrhage, oedema and suppurative or lymphoplasmacytic inflammation. A silver stain (WS, ArtisanLink DAKO) was performed on the kidneys to demonstrate the presence of spirochaetes, and where available from the necropsy reports we included results of previous silver stains (WS) on the given animal's liver.

Immunohistochemistry

IHC using rabbit anti-OMV2177 raised against LPS of *L. interrogans* serovar Copenhageni (kindly provided by Prof. Dr. Simone Schuller, Vetsuisse Faculty, Berne) [88] was performed in 64 cases on sections of liver and kidney and in 35 cases on kidney alone. The

majority of renal samples that were negative or inconclusive by IHC using OMV2177 (n=63) were tested with an antibody raised in rabbits against LipL32 (kindly provided by Dr. Jarlath Nally, USDA Agriculture Research Service, Aimes, Iowa, USA), a major outer membrane protein present on all pathogenic *Leptospira*.

To perform IHC, standard protocols were applied. Briefly, 2 to 3 µm sections of tissue were cut from the paraffin blocks and placed on positively charged glass slides (Bond Plus slides, Leica Microsystems Ltd, UK) and dried over night at 37 °C. Paraffin was removed from sections with xylene and ethanol, and slides were subsequently rehydrated using Leica autostainer© (Leica Microsystems Ltd, UK). For antigen retrieval we pre-treated the samples with acidic buffer (DakoCytomation Target Retrieval Solution Citrate pH 6 (10x), S2369) for 20 min in a pressure cooker (Pascal DakoCytomation) at 98 °C. After every step the slides were rinsed for 10 min with wash buffer (Dako Wash Buffer 10x, Dako S3006). To avoid nonspecific staining of tissue we applied a peroxidase block (Dako, S2023) for 10 min at RT, prior to incubation for 30 min at RT with the primary antibody rabbit anti-OMV2177 at a dilution of 1:2000. As secondary antibody kit the EnVision Rabbit was used (EnVison^{+}Labelled Polymer System-HRP anti Rabbit, Dako K4003) for 30 min at RT. AEC (amino ethyl carbazol, Dako K3464) was used as chromogen for 10 min at RT, and slides were counterstained with haematoxylin. IHC for LipL32 was conducted on BOND-III Fully Automated stainer (Leica Microsystems Ltd, UK) using citrate buffer pH 6.0 for 20 min in heat for antigen retrieval and an antibody concentration of 1:1250 for 60 min at RT. As positive controls for both antibodies we used the kidney tissue from: (i) a dog with confirmed leptospirosis (via qPCR), and (ii) a rat experimentally infected with *L. interrogans* serovar Copenhageni. Negative controls included omission of primary, secondary and both antibodies, as well as renal tissue from a dog with unrelated disease. An isotype antibody control was not performed.

Molecular Analysis

Extraction

Two protocols were applied to extract DNA from FFPE material. Firstly, 5-10 µm thick sections (not more than 25 mg) of the tissue block containing the kidney were prepared and collected in a tube. Then we proceeded with either of the two following protocols.

A) For our first approach we used the QIAamp® DNA FFPE Tissue Kit (Qiagen). According to manufacturer's instruction we mixed and vortexed the tissue with 320 µl deparaffinization solution (Qiagen). After 3 min incubation at 56 °C 180 µl lysis buffer ATL was

added. 1 min centrifugation at 10,000 RPM separated the mixture and 20 µl Proteinase K added to the lower phase initiated the lysis process. Incubation for an hour each at 56 °C and 90 °C was followed by transferring the lysed section into a new tube and the sample was treated according to the steps in the manufacturer's instruction. Finally, the nucleic acid was diluted in 75 µl elution buffer (ATE).

B) Alternatively, we used the DNeasy® Blood & Tissue Kit (Qiagen) to perform nucleic acid extractions from FFPE tissue of the kidney, with modifications for formalin-fixed samples as recommended by the manufacturer. Briefly, to remove the paraffin the sample was mixed with xylene, and subsequently ethanol to remove the xylene. To destroy formalin-induced crosslinking, the tissue was incubated at 98 °C for 15 min. After this pre-treatment of FFPE tissues, 180 µl lysis buffer ATL was added to the tissue pellet. To facilitate lysis, we added 20 µl Proteinase K and kept the homogenate at 56 °C until the tissue was completely lysed (approximately 1 hour). Subsequent steps were performed according to the manufacturer's protocol and nucleic acids were eluted in 200 µl elution buffer (AE).

DNA Quantification

After both protocols, microvolume spectrophotometry (NanoDrop™ Thermo Scientific™) facilitated measurement of the concentration of nucleic acid of the samples based on the intrinsic absorptivity properties at the characteristic peak at 260 nm.

qPCR

The real-time PCR (qPCR), previously validated on fluids [102] and foetal tissues [101] was carried out on 94 samples using primers LipgrF2 (5'-CGCTGAAATGGGAGTTCGTATGATTTCC-3') and LipgrR2 (5'-GGCATTGATTTTTCTTCYGGGGTWGCC-3') (Eurofins Genomics UK Limited). A total of 0.2 µl (forward primer) and 1.2 µl (reverse primer) of a 100 µM solution were added to a reaction mix. This setting used the probe LipgrP2 (5'-AGGCGAAATCGGKGARCCAGGCGAYGG-3') (Eurofins Genomics UK Limited) with a volume of 0.8 µl. The FastStart Universal Probe Master (Rox) (Roche, USA) containing TaqMan DNA polymerase, reaction Buffer, nucleotides (dATP, dCTP, dGTP, dUTP), and a reference dye was used according to the manufacturer's protocol and using 5 µl of DNA template in 15 µl reaction (primer, probe, FastStart mix, water). A 7500 Fast Real-Time PCR System (Applied Biosystems, software version 2.0.6) was used with MicroAmp Fast optical 96-well plates and Optical Adhesive Films (Applied Biosystems). Amplification was performed using 20 µl/well and the following thermal cycle: 95 °C 10 min,

and 40 cycles: 95 °C for 15 s, 60 °C for 1 min. For technical replication we performed qPCR in duplicates and corrected results for amplification efficiency. Controls included a negative control without template and positive controls from fresh and FFPE renal tissues of previously confirmed (qPCR) canine diagnostic samples.

Qubit© Assay

To evaluate how much DNA was in the samples we compared fluorometry (Qubit© Assay) versus microvolume spectrophotometry (NanoDrop™ Thermo Scientific™) for DNA quantification. A Qubit© assay working solution was created by diluting Qubit© reagent(dye) 1:200 with Qubit© buffer (3000 µl and 15 µl respectively). The standard (n=2; volume 190 µl) was mixed with 10 µl working solution and the samples (n=12; volume 199 µl) with 1 µl working solution and afterwards incubated for 2 min at RT. Measuring the DNA absorbance at 260 nm was facilitated with a Qubit© 2.0 Fluorometer.

Internal Positive Control (IPC) Dogs

To assess if there was amplifiable DNA in these samples we included an internal positive control, i.e. canine DNA. Primers targeting dog ribonuclease P/MRP subunit p30 (RPP30) gene were designed in-house using the Primer 3 web application (https://primer3.ut.ee/) and were then validated using the standard curve method and shown to have an efficiency of 96.5 % (personal communication with John A. Browne). For each dog 1.2 µl forward primer (5'-CGAGGTGTGTGCTTTGAACT-3') and reverse primer (5'-TGAGGGCATTGGAAATTGTGT-3') (Sigma-Aldrich, United Kingdom) were mixed with 10 µl SYBR® Green qPCR ReadyMix™ (Sigma-Aldrich, United Kingdom) and 5 µl of extracted sample DNA. A 7500 Fast Real-Time PCR System (Applied Biosystems, software version 2.0.6) was used with MicroAmp Fast optical 96-well plates and Optical Adhesive Films (Applied Biosystems). Amplification was performed using 15 µl/well and the following thermal cycle: 95 °C for 2 min, and 40 cycles: 95 °C for 15 s, 60 °C for 1 min. We performed the assay in duplicates for technical replication and corrected results for amplification efficiency.

SPUD Assay

To investigate if contamination within these samples causes inhibition of amplification, we performed a SPUD assay which used a sheep RPP30 probe assay with a Taqman qPCR protocol, as described above. Slight alterations regarding the volume were made, i.e. 12.5 µl FastStart Universal Probe Master (Rox) with each 0.25 µl forward and reverse primer, 0.375

µl prober and 1 µl sheep DNA were mixed with different dilutions (added volumes were none, 1.0, 2.0, 3.0, 4.0 and 5.0 µl respectively) of extracted canine DNA FFPE samples.

Genomic DNA Clean-Up

To eliminate inhibiting substances within the extracted DNA samples the clean-up kit NuceloSpin© gDNA Clean-up US (Machery-Nagel. Germany) was used on 16 samples according to the manufacturer's manual.

PCR

A diagnostic sample (renal tissue of a dog) previously identified as *Leptospira* positive (using qPCR, cycle threshold (CT) value 34) underwent end point PCR analysis with three different primer pairs (Table 1) for further exploration [121]. The reaction mix for PCR consisted of 25 µl PCR Master Mix (Qiagen), 2.0 µl of forward primer (10 pmol/ µl stock), 2.0 µl of reverse primer (10 pmol/µl stock), 16 µl nuclease-free water and 5 mL DNA template (derived from renal tissue), for a total reaction volume of 50 µl. The reaction mix was run for 5 min at 95 ^{o}C, followed by 30 cycles of 30 s at 95 ^{o}C, 30 s at 55 ^{o}C and 30 s at 72 ^{o}C on a DNA Engine Dyad Peltier Thermal Cycler (Biorad) for Intergroup A (see table below). For Intergroup B and Kirschner a TM of 58 ^{o}C was used. After 30 cycles an extension period of 7 min at 72 ^{o}C followed.

Products were separated via gel electrophoresis and stained with Ethidium Bromide and visualised under UV light. As the sample was considered to have a low abundance of leptospiral genomic material (CT = 34) and the protocol may also have to be optimised further, the PCR was re-run using DNA derived from pure culture.

In a second optimisation, pure cultured *L. interrogans* DNA was used (concentration of 37.75 ng/µl). The isolated DNA was then adjusted to 10 ng/µl with nuclease-free water. This reaction mix consisted of 25 µl MasterMix (Qiagen), 1 µl of forward primer (10 pmol/µl stock), 1 µl of reverse primer (10 pmol/µl stock), 18 µl nuclease-free water and 5 ml DNA (10 ng/µl), for a total reaction volume of 50 µl. A temperature gradient was used for all three primer pairs in six different annealing temperatures, i.e. 39.6, 42.5, 48.2, 54.9, 59.2, and 61.0 ^{o}C so that the optimal annealing temperature could be established.

The PCR products were visualised with molecular weight markers Invitrogen Low DNA mass ladder, (10068-013) and Promega 100 bp DNA ladder (G210A) on a 2 % agarose gel and documented with a Multi DOC imaging system (UVP).

Table 1. Species-specific primer sequences for detection of *Leptospira* via PCR

Primer	Oligonucleotide sequence (5' → 3')
Intergroup A fwd	CTACTGGCGGCTTGATCAAC
Intergroup A rev	CTGGATCTGTTCCGTCTGCGATC
Intergroup B fwd	CTTGATAGAACCACTGGTGGTGCC
Intergroup B rev	CTGGATCGGTTCCATCGCTCAG
Kirschner fwd	CGGTTTGATCAATGCGAGAAGCACC
Kirschner rev	TTGGATCCGTTCCGTCTGCGATT

Touchdown PCR

The next step was to optimise the PCR further using a touchdown PCR, which started at a high annealing temperature and then decreased by 1 °C per cycle until it reached the optimal annealing temperature (in terms of quantity) which was 39.6 °C for the remaining 20 cycles, using the same master mix as was used previously. For details see table 2 below.

Table 2. Settings for touchdown PCR

	Temperature	Duration
Initialisation	95 °C	5 min
For 10 cycles	95 °C	30 sec
	61 °C (decrease by 1 °C per cycle)	30 sec
	72 °C	30 sec
For 20 cycles	95 °C	30 sec
	39.6 °C	30 sec
	72 °C	30 sec

Results

Confirmation of *Leptospira* sp.

The combination of staining methods (IHC) and molecular tests (qPCR) confirmed the presence of leptospiral antigen (IHC) or genomic material (qPCR) in 73/99 (73 %) of kidneys with suspected leptospirosis. A similar detection rate was achieved where dogs were either diagnosed with exudative GN (74 %; 37/50) or with 'classical' leptospirosis based on previously defined clinicopathological criteria (73 %; 36/49). A detailed description of those is given in Fig. 1.

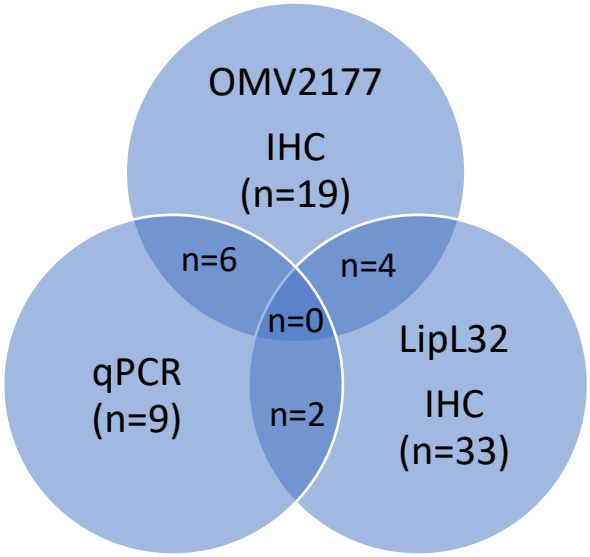

Figure 1. This figure illustrates the number of samples detected by the different tests. The majority of samples (n = 61) were detected by one test only. Twelve cases were confirmed by a combination of two methods. No case was confirmed by more than two methods.

Immunohistochemistry

The presence of leptospiral antigen was confirmed in 39 of 58 tested cases (67 %) using LipL32 antibody for IHC. These included 23/33 (70 %) exudative GN and 16/25 (64 %) classic leptospirosis presentations. Positive brown granular labelling was seen in the proximal tubular epithelial cells, cells in the glomeruli most likely mesangial cells or admixed with the erythrocytes, cells located in the interstitium for example spindle shaped cells interpreted as fibrocytes and macrophages or macrophages in the vessels mostly in the subcapsular veins.

In general, only a small amount of brown labelling was seen associated with acute tubular necrosis and minimal antigen was present in the cases with chronic interstitial

nephritis. Additionally, dense fine granular brown lining of the apical borders of tubular epithelial cells in multifocal small groups of cortical tubules was found (Fig. 2).

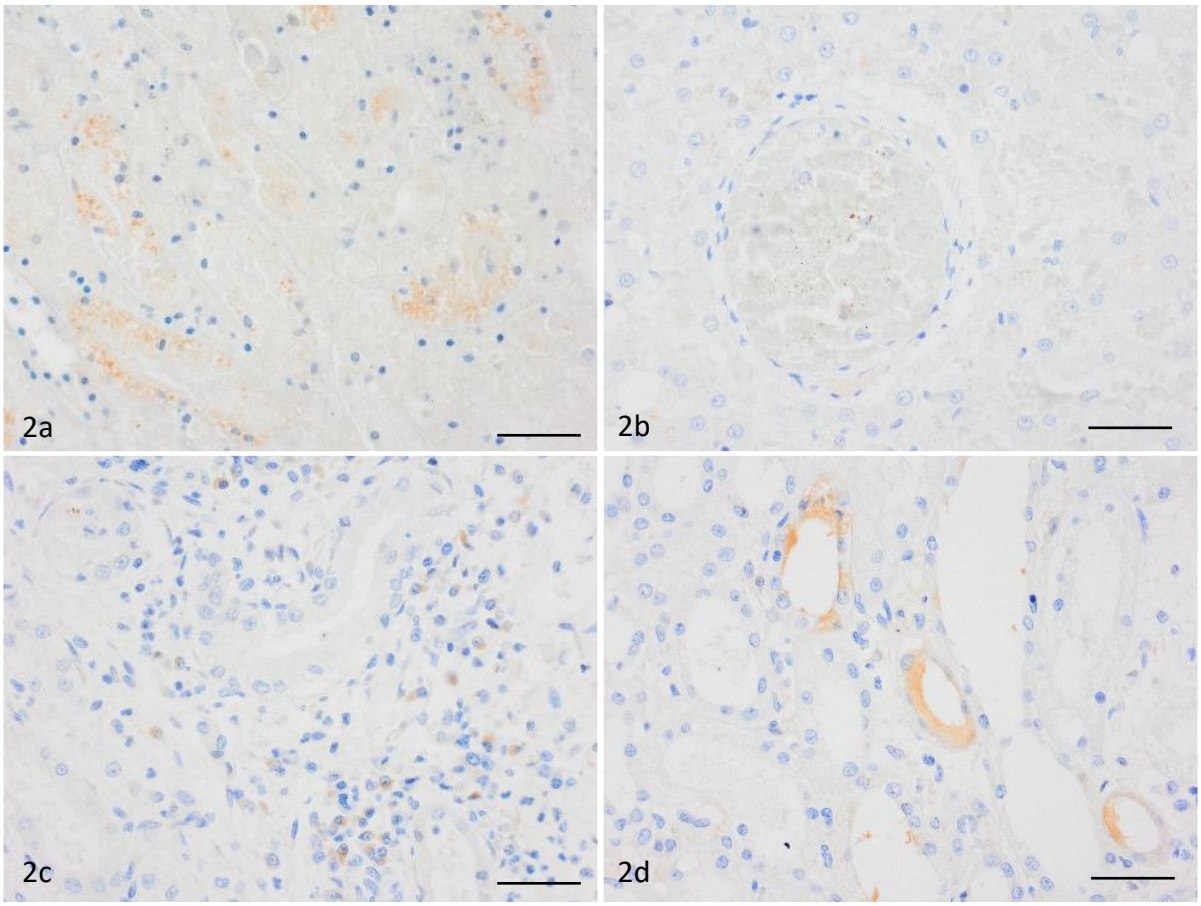

Figure 2. Immunohistochemistry using anti-LipL32 in canine leptospirosis cases. (a) Positive brown granular labelling was seen in the proximal tubular epithelial cells. (b) Brown multifocal and pinpoint immunolabelling is present within a glomerulus obliterated by erythrocytes. (c) Positive brown granular labelling was seen in interstitial cells, likely fibrocytes and macrophages. (d) A small amount of brown labelling was seen associated with acute tubular necrosis. Bar 50 μm. IHC LipL32.

Twenty-nine of the 99 cases (29 %) could be confirmed using OMV2177 antibody. This included 10 cases of the 50 exudative GN (20%) and 19 of the 49 (39 %) cases of classical leptospirosis. By combining the results of IHC using these antibodies a detection rate of 64/99 (65 %) was achieved. Similar labelling patterns were found with both the OMV2177 and LipL32 antibodies (Fig. 3).

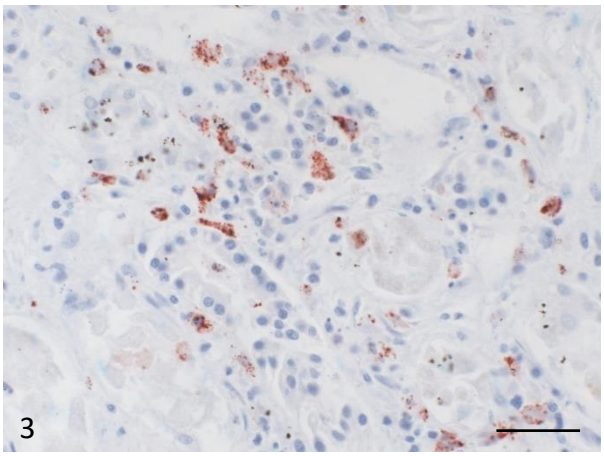

Figure 3. Immunohistochemistry using anti-OMV2177 in canine leptospirosis cases. Positive red granular labelling was seen in interstitial cells, likely fibrocytes and macrophages. Bar 50 μm. IHC OMV2177.

Overall, we observed that acute lesions had less detectable antigen visible with IHC.

Extraction and DNA Quantification

After both DNA extraction protocols, NanoDrop™ Thermo Scientific™ DNA concentration ranged from 4.94 to 1,226.1 ng/μl (Appendix, Table A1).

Comparing the two protocols QIAamp® DNA FFPE Tissue Kit (Qiagen) and DNeasy® Blood & Tissue Kit (Qiagen), we observed that samples extracted with the FFPE specific kit provided higher nucleic acid concentrations (Table 3), but the absorption curve featured an elevated trough around 230 nm (Appendix, Figure S1). This is indicative of contamination, for example by sugar, salts, or organic solvents. Alternatively, samples extracted with the fresh tissue kit featured lower nucleic acid concentrations (Table 3), but the absorption curve presented a clean peak at 260 nm (representing DNA absorption) without second peaks, shifted or elevated troughs, which is consistent with less contamination. Nevertheless, a subsequent qPCR only amplified leptospiral DNA from the FFPE kit, which is why this extraction method was used.

Table 3. Comparison of nucleic acid concentration of samples extracted with FFPE Tissue Kit and Blood & Tissue Kit measured with NanoDrop.

Sample	Nucleic Acid (ng/µl) QIAamp® DNA FFPE Tissue Kit (Qiagen)	Nucleic Acid (ng/µl) DNeasy® Blood & Tissue Kit (Qiagen)
1	185.76	72.37
2	133.74	25.63
3	186.58	87.01
4	40.61	7.79

Qubit© Assay

Expanded DNA measurement disclosed that our extracted samples contained very low amounts of DNA. Fluorometry measured a median of 63.5 ng/µl with the Qubit© Assay, which was significantly less compared to a median of 458.9 ng/µl assessed with the spectrometry (NanoDrop) method (for details see table below).

Table 4. Nucleic acid concentration in ng/µl measured with Qubit© and NanoDrop.

Sample ID	Qubit©	NanoDrop
1	14.1	1,226
3	0.554	609
5	26.5	416
7	25.4	359
9	27.3	453
11	13	238
13	27.6	181
15	10.4	445

qPCR

Previous qPCR conducted by A. Ahmed at the WHO/FAO/OIE and National Collaborating Centre for Reference and Research on Leptospirosis, Amsterdam, Netherlands, yielded a positive signal for 16/31 (52%) samples. Thirteen of those were cases of exudative GN, and 3 were classical cases.

An additional 80 samples were tested that included repetitive samples from the reference laboratory. Only two samples delivered a positive result, both were cases of

exudative GN, and samples were extracted with the FFPE Tissue Kit. One of these samples had already tested positive, resulting a total of 17 confirmed cases by qPCR.

Subsequently we tried to improve the sensitivity of qPCR on FFPE material (see below).

Internal Positive Control Dogs

As part of the internal positive control, the control wells with canine DNA and research samples presented amplifiable canine DNA and no amplification of canine DNA was measured in the negative control well, i.e. no template sample. This assay confirmed the presence of amplifiable DNA within samples and the performance of the assay.

SPUD Assay

To evaluate if potential contaminants within the samples caused inhibition of amplification, we performed a SPUD assay with ovine DNA and increasing volumes of the samples. The smallest amount of sample material (i.e. 1 µl extracted canine tissue) admixed with ovine DNA inhibited sheep DNA amplification, indicating a significant amount of amplification inhibitors within the study samples.

Genomic DNA Clean-Up

In a trial to eliminate amplification inhibitors we performed a clean-up on a group of samples. The DNA concentration (ng/µl) after the cleaning was significantly lower than before the cleaning step (Table 5). A following TaqMan qPCR did not generate unambiguously positive results and was considered negative for all tested samples.

Table 5. Nucleic acid concentration in ng/µl measured with Qubit©.

ID	Untreated Sample	Cleaned Sample
1	101.8	14.1
2	41.2	NA
3	47.0	0.6
4	74.6	NA
5	61.0	26.5
6	69.6	NA
7	47.4	25.4
8	66.0	NA
9	76.4	27.3
10	47.8	NA
11	8.0	13.0
12	133.8	NA
13	NA	27.6
14	NA	NA
15	NA	10.4
16	NA	NA
Average	**64.6**	**18.1**

*NA = not available

PCR

As the qPCR assay did not amplify any *Leptospira* DNA in the vast majority of samples, we explored the usefulness of a conventional PCR with previously confirmed *Leptospira* positive canine renal tissue (outside of the study group). PCR did not yield any visible products on gel electrophoresis for any of the primer pairs (Fig. 4). Expected sizes of amplifiable fragments were 396 base pairs (bp) for Interrogans group A primers, 406 bp for Interrogans group B, and 389 bp for Kirschner primers [121].

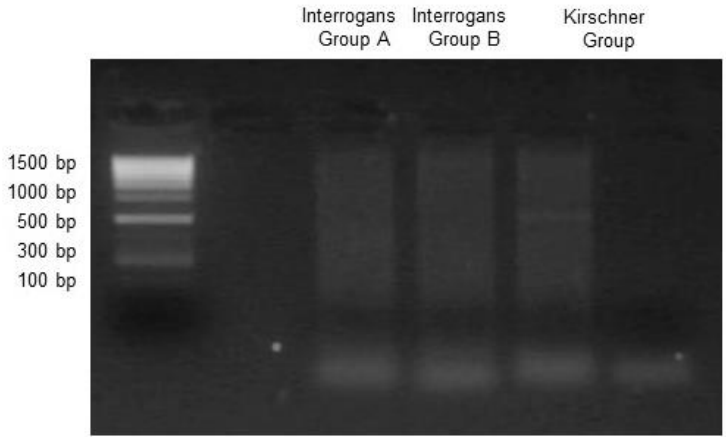

Figure 4. Gel electrophoresis of PCR on study samples. Primer pairs of Interrogans group A, Interrogans group B, and Kirschner did not yield any product.

A subsequent trial of optimisation with DNA of pure cultured *L. interrogans* with the same three pairs of primers and a combination of annealing temperatures did not amplify *Leptospira* DNA without additional non-specific amplification and was for that reason not considered a sufficient method of detection.

More specifically, PCR with Interrogans group A primer pairs amplified DNA at the lower annealing temperature spectrum, i.e. 39.6, 42.5, and 48.2 ^{o}C. The PCR products visualised with molecular weight markers showed two bands, one of the correct size (396 bp) and one non-specific band (around 950 bp) (Fig. 5A). The Interrogans group B primers did not yield any product for any annealing temperature (Fig. 5B). Finally, primers for Kirchner displayed only a non-specific band (around 700 bp) for the lower annealing temperatures, i.e. 39.6, 42.5, and 48.2 ^{o}C. (Fig. 5C).

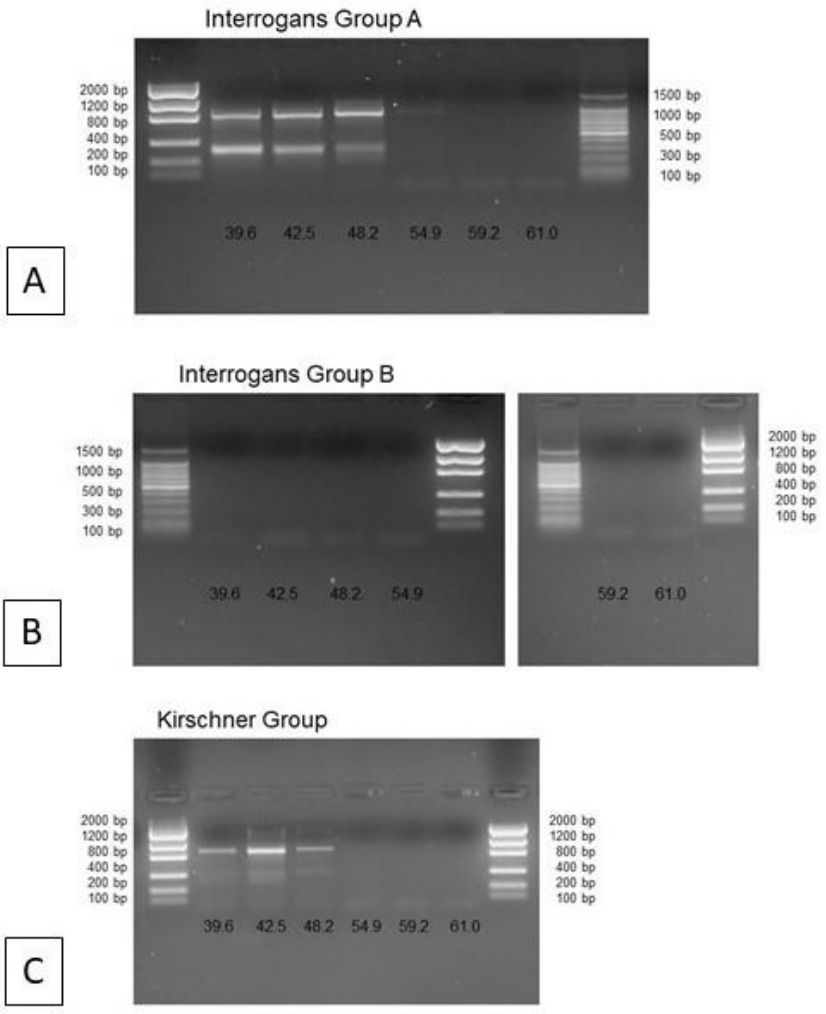

Figure 5. Gel electrophoresis of PCR on cultured *L. interrogans* with annealing temperature spectrum from 39.6 to 61.0 °C. (A) Interrogans group A primer pairs amplified two bands of DNA at 39.6, 42.5, and 48.2 °C; one of the correct size (396 bp) and one non-specific band (around 950 bp). (B) Interrogans group B primers did not yield any product for any annealing temperature. (C). Kirchner primers displayed only a non-specific band (around 700 bp) for annealing temperatures, i.e. 39.6, 42.5, and 48.2 °C.

In conclusion, PCR assays with three different primer pairs and a variety of annealing temperatures did not detect *Leptospira* in study samples or extracted *Leptospira* samples from pure culture and were thus considered not suitable for this study.

Touchdown PCR

Afterwards we ran a touchdown PCR with varying annealing temperatures to increase the specificity of the PCR assay [122]. We used DNA of pure cultured *Leptospira interrogans* in a touchdown PCR setting with the primers for Interrogans group A and Kirchner. This still

generated non-specific DNA amplification next to *Leptospira* specific bands (visualisation not shown) and thus exploring this method was discontinued.

Exudative GN in Dogs Associated with Leptospirosis

Thirty-seven dogs with exudative GN tested positive for *Leptospira* antigen by IHC. Details on the signalment, clinical signs and pathological findings of this previously unreported disease presentation are described below.

Signalment and Clinical Signs

Affected dogs were 2 months to 14 years old, with an average of 5.32 ± 4.07 years, and median of 6 years. Eleven dogs were younger than 6 months. The sex was given for 36 dogs the majority of which were male (n = 21, 58.3 %), of which six were castrated. Fifteen dogs were female (41.7 %), of those six were neutered. Twenty-three breeds were represented, the most common being the Labrador Retriever (n=7) followed by mixed breed dogs (n=6) and Golden Retrievers (n=2). Large sized dogs were most common (n=16), followed by medium sized dogs (n=10) and small breed dogs (n=4). The canton of Zurich was home to most cases (n=18) followed by the canton of Aargau (n=7) and the canton of Bern (n=4). Late summer and early autumn were the seasons when most cases were recorded.

Dogs most often presented clinically with vomiting (n = 21), which was occasionally bloody, and icterus (n = 20). Less frequently observed was diarrhoea (n = 14), often associated with blood, anuria (n = 10), anorexia (n = 9) haematuria (n = 7), apathy (n = 7) and dyspnoea (n = 5). Fever was only reported in one dog. Haematological tests were available in 12 cases and indicated anaemia (n = 4) and/or thrombocytopenia (n = 9). Twenty-six cases provided biochemistry results and azotaemia was commonly reported (n = 19) whereas an increase in liver enzymes, i.e. ALT, AP, and total bilirubin were less common (n=8). None of the dogs survived longer than ten days after initial presentation of clinical signs and dogs either died or were euthanised.

Pathological Changes and Warthin-Starry Stain

Macroscopically, petechial to larger ecchymotic renal cortical haemorrhages were seen in 16 cases, six had a swollen and discoloured kidney, and the remaining 15 had no gross changes.

Microscopically, exudative GN affected more than 50% of the glomeruli (diffuse lesion) and most of the glomerular tuft was involved (global lesion) in the process. It was characterised by large amounts of fibrin, abundant extravasated erythrocytes and neutrophils

in the glomerular tuft, mesangium and particularly expanding the urinary spaces, compressing the glomerular tuft and ultimately obscuring the glomeruli (Fig. 6A). In some glomeruli activated mesangial cells were visible. Some glomerular tufts were necrotic, and the beginning of some crescent formation (fibrocelullar) was observed. The capillaries of the glomeruli were often markedly dilated by erythrocytes. The incoming (afferent arterioles) and outcoming vessels (efferent arterioles) were often occluded with fibrinous exudate (Fig. 6B). Often a periglomerular inflammation with extravasated erythrocytes and neutrophils was visible (Fig. 6C).

Using Periodic acid–Schiff (PAS) staining the fibrinous character of the exudate was confirmed and the glomerular basement membranes that could be assessed were not thickened (Fig. 6C).

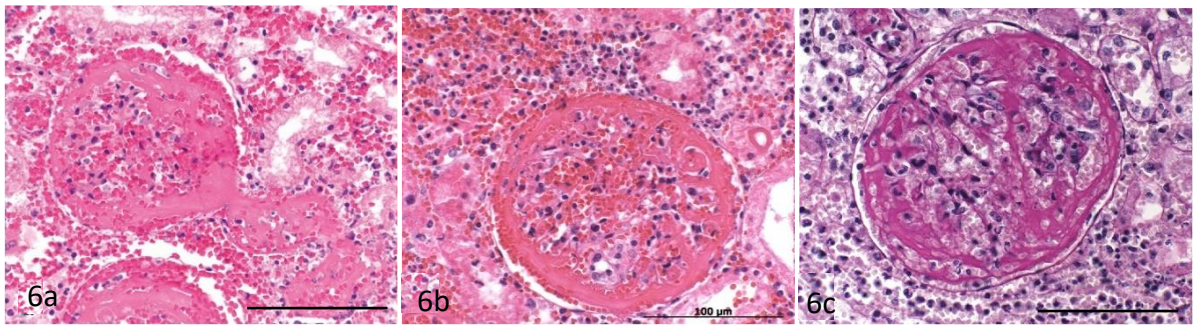

Figure 6. Kidney, canine leptospirosis with exudative glomerulonephritis. (a) Large amounts of fibrin and abundant extravasated erythrocytes are expanding the glomerular tuft and the urinary space. The glomerular vessels are occluded by fibrinous exudate and there is interstitial haemorrhage. Bar 100 µm. Haematoxylin and eosin stain (HE). (b) There is periglomerular neutrophilic inflammation and acute tubular necrosis. Bar 100 µm. HE. (c) The fibrinous character of the exudate was confirmed by PAS staining. Bar 100 µm. PAS.

Acute tubular necrosis with degeneration and/or necrosis of tubular epithelial cells, pyknotic nuclei and hypereosinophilic cytoplasm as well as sloughing of tubular epithelial cells into the lumen of tubules was frequently seen (Fig. 7A). Tubular regeneration characterised by mitotic figures and/or double nucleated tubular epithelial cells was only occasionally observed.

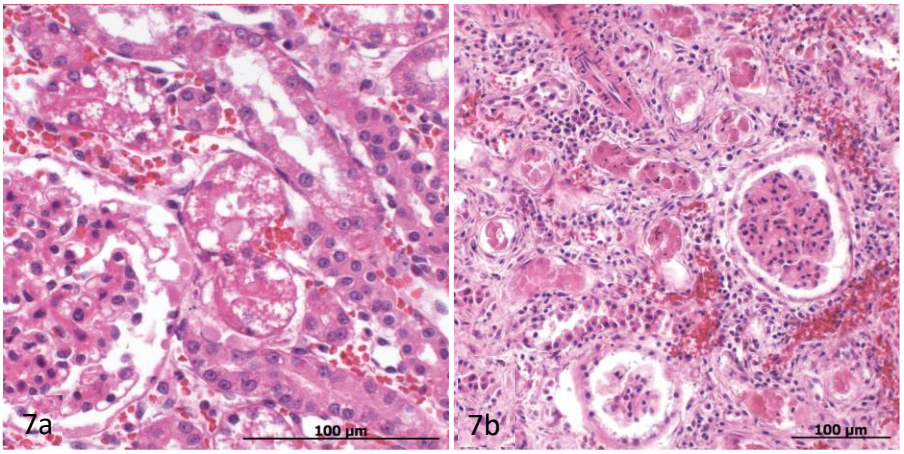

Figure 7. Kidney, canine leptospirosis with exudative glomerulonephritis. (a) Acute tubular necrosis characterised by hypereosinophilic cytoplasm, pyknotic nuclei, and sloughing of tubular epithelial cells into lumens. Haematoxylin and eosin stain (HE). (b) Marked distention of the interstitium by lymphocytes, fewer plasma cells and macrophages. HE.

In addition, acute multifocal to coalescing haemorrhages were seen in the interstitium in about two thirds of the cases (Fig. 6A, B). Interstitial nephritis was diagnosed in 29 cases. Infiltration of neutrophils (n = 19) was associated in some cases with varying amounts of lymphocytes and plasma cells in the interstitium (n = 6). Lymphoplasmacytic infiltrate was only present in another 10 cases (Fig. 7B). Varying numbers of tubules contained bright eosinophilic homogeneous (protein) casts in 10 cases.

Fibrinoid degeneration of the walls of smaller vessels in the interstitium was seen in four cases and thrombosis in another two. Lesions in the renal pelvis included mild, mostly lymphocytic infiltration (n = 6) or acute haemorrhage (n=3).

Overall, 20 of the 37 confirmed exudative GN cases were positive when examined using a WS stain (54 %). The argyrophilic bacteria were mostly found in the proximal tubular epithelia. Most of them were degraded and few had the typical cork-screw morphology (Fig. 8A, B).

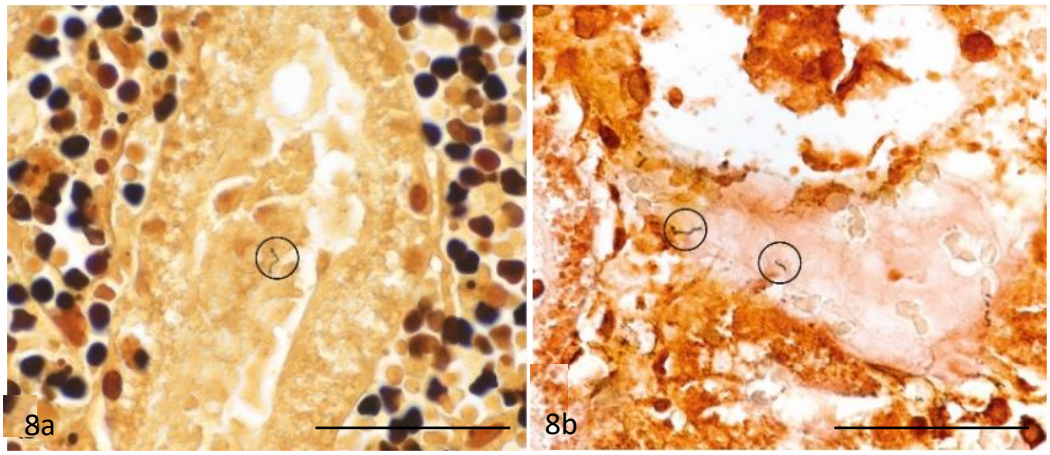

Figure 8. Kidney, canine leptospirosis with exudative glomerulonephritis (A) A black argyrophilic leptospiral organism with the "Kleiderbügel" shape is visualised in the tubular epithelial cells and highlighted by a black circle. Bar 100 µm. Warthin-Starry stain (WS). (B) Black argyrophilic leptospiral organisms highlighted by black circles are observed in the lumen of a necrotic tubule. Bar 100 µm. WS.

A summary of the additional histopathological findings is shown in Table 6.

Table 6. Microscopically observed lesions associated with cases of exudative glomerulonephritis in dogs with leptospirosis.

Microscopic changes	**Exudative GN (n= 37)**
Acute tubular necrosis	32 (87 %)
Tubular regeneration	5 (14 %)
Protein casts in tubules	10 (27 %)
Suppurative interstitial nephritis	19 (51 %)
Lymphoplasmacytic interstitial nephritis	16 (49 %)
Interstitial haemorrhages	25 (68 %)
Fibrinoid degeneration of small vessel walls	4 (11 %)
	Lung (n= 30)
Pulmonary haemorrhage	27 (90 %)
	Liver (n= 33)
Hepatocyte dissociation	27 (82 %)

Discussion

This study describes exudative GN in dogs for the first time and demonstrates an association between the glomerular changes and an infection with *Leptospira* species.

IHC yielded the highest percentage of leptospiral positivity in this study, indicating this as the most sensitive method when using archived FFPE material. LipL32 is an outer membrane protein that is expressed by all pathogenic *Leptospira* species whereas OMV2177 antigen is targeting LPS of *L. interrogans* serovar Copenhageni. Antibodies against the latter did not perform well in this study, especially in the cases of exudative GN, which is why we suspect the *Leptospira* serovar causing this lesion differs from Copenhageni (*Leptospira interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni) and related serovars. While we were able to identify intact leptospires within renal tubules, the detection of fragmented or phagocytosed leptospires might be limited when focusing on a single surface protein. Moreover, the best reactivity in IHC is achieved with an antibody that is raised against the LPS of the serovar with which the animal is infected. The lack of use of labelling antibodies against specific antigens in this study has most likely resulted in some cases not being detected. Further limiting conditions to detect *Leptospira* with IHC specifically in cases of exudative GN include the large amount of extravasated erythrocytes, neutrophils, and fibrin which could potentially have obscured or degraded antigens. On top of that, a low number of pathogens in the examined tissue, as can be seen in acute disease, likely resulted in decreased detection rates and thus failure to confirm leptospiral infection. The postmortem detection of *Leptospira* can be enhanced by testing multiple organs, such as lungs, liver, and kidney [123]. However, in the present study, the examination of the liver using IHC and WS did not provide additional information compared to the kidney (personal communication with Monika Hilbe) and as a result only renal tissue was included in the analysis.

As briefly mentioned above, the lower detection rate of exudative GN cases by OMV2177 indicate that the causative serovar differs from Copenhageni and related serovars. This is further supported by the fact that cases of exudative GN, and leptospirosis in general, have decreased since canine vaccination protocols were updated from the previous bivalent vaccination (Icterohaemorrhagiae and Canicola) to the current four serogroups (Icterohaemorrhagiae, Canicola, Australis, Grippotyphosa). The current vaccine covers the most common serovars in Europe: Australis, Bratislava, Copenhageni, Canicola, Grippotyphosa, and Icterohaemorrhagiae [115].

Real-time PCR can be used to confirm various pathogens in FFPE [124, 125], and the reference laboratory yielded positive results for *Leptospira* sp. in 52 % of the examined cases while a subsequent, more extensive run only confirmed leptospirosis in 3 % of the samples, even though the same detection protocol, including probe, primers, and polymerase were used. We performed extensive problem analysis and used different methods for extraction and molecular techniques to optimise detection of *Leptospira* sp. and we identified several sources that may have created this discrepancy: (1) low DNA content, (2) amplification inhibitors, and (3) sample quality.

(1) We assumed that the initial leptospiral DNA content within samples was already very low, often indicated by individual cells in IHC that showed labelled positively. Sensitive DNA measurement with Qubit© assay [126] confirmed overall low DNA yield which can be caused by general low leptospiral DNA content, age of samples, fixation, or suboptimal extraction. Furthermore, we could not improve DNA content by using different extraction methods. Comparing a modified extraction kit for fresh tissue and blood (used by the reference laboratory) [120] with a commercial FFPE-specific kit showed higher nucleic acid concentration with the FFPE-specific kit, although remaining contaminations were indicated. Only the FFPE kit yielded positive qPCR results, making it the chosen method for the ongoing study. (2) Another major concern was the presence of amplification inhibitors, as confirmed by the SPUD assay. A trial to clean the samples only yielded in a lesser amount of DNA and did not produce any positive results, which is why a clean-up of all samples was not initiated. (3) It is well established that formalin fixation modifies and destroys genetic material, which makes it less accessible for amplification compared with fresh samples [127-129]. Additionally, the time lag between when the qPCR was performed in the reference laboratory (in 2019) and the later run (2021/2022) might have contributed to the loss of genetic material.

An original objective of this thesis was to sequence amplified bacterial genomic products to investigate if a specific, potentially new, serovar was causing these unusual renal lesions in dogs. As none of the methods deployed in this study resulted in an unambiguous positive result, a last effort to identify the leptospiral species in an external institute (the reference laboratory) was attempted. In one sample of FFPE tissues from four cases of exudative GN, *L. interrogans* was identified using SybrGreen PCR. However, poor DNA yield retrieved from the FFPE tissues hampered interpretation of the results and further analysis was terminated (personal communication with A. Ahmed).

Exudative GN is characterised by marked fibrin exudation into the urinary space, as it occurs in severely damaged glomeruli with increased permeability of the glomerular basement membrane. This leads to proliferation of glomerular epithelial cells which manifest as a glomerular crescent, and is often accompanied by infiltration of macrophages and neutrophils [7, 114]. In humans, crescents are most commonly seen in cases of rapidly progressive GN and are indicative of severe glomerular damage rather than being pathognomonic for any specific disease [40]. Crescent formation is rare in dogs (and other domestic animals) but is described in pigs with porcine dermatitis and nephropathy syndrome, where it is understood to be an immune-complex mediated lesion characterised by fibrino-necrotising GN and systemic vasculitis linked to porcine circovirus 2 infection [7, 40, 41]. While the use of the term exudative GN in dogs has fallen out of favour in the current literature [7, 130], older publications describe exudative GN and report it to closely mimic chronic interstitial nephritis clinically, with polydipsia, polyuria and uraemia [114]. Furthermore, glomerular abnormalities similar to those reported in this study have been rarely documented previously. These report findings such as glomerular hyperaemia, mesangial matrix expansion, and marked fibrinoid change, necrosis, or sclerosis, but only in a small number of dogs with *Leptospira interrogans* Australis serogroup infection [131].

Typical microscopic renal findings in acute leptospirosis in dogs are tubular necrosis and/or interstitial or tubulointerstitial nephritis [53, 132, 133] – all changes that were observed in cases with exudative GN, and further support the link between leptospirosis and exudative GN. Classic hepatic lesions include hepatocellular dissociation, single cell necrosis and mitotic figures [134] which were also common findings in the dogs in this study. Frequently observed pulmonary lesions in all study dogs included multifocal moderate pulmonary haemorrhage, as part of widespread haemorrhages of acute leptospirosis as described in experimentally infected dogs with serovar Pomona [135]. These moderate lesions however, must be differentiated from the recently described leptospiral pulmonary haemorrhage syndrome in dogs which is much more severe and diffuse [117].

PAS staining confirmed an intact and thin glomerular basement membrane in the present cases of exudative GN. This suggests that the exudative changes were caused by a marked increase in vascular permeability within the glomerular capillaries, rather than by circulating immune complexes as their deposition would potentially cause thickening and loss of basement membrane continuity. No signs of thrombotic microangiopathy were observed

within the main organs and only few intravascular renal thrombi (n=2) and occasional (n=4) mild multifocal fibrinoid necrosis of renal vessels was seen, which is known to occur in dogs with acute fatal leptospirosis [136]. Marked increased vascular permeability in leptospirosis can be explained by two mechanisms: (1) the disruption of endothelial cell adherens junctions, which is mediated by VE-cadherin, an important receptor for pathogenic leptospires, and (2) the alteration of the cell membrane, leading to altered permeability [118, 137, 138].

The gold standard for diagnosing glomerular disease in human medicine comprises examination of sections with light microscopy (including a specific panel of histochemical stains), immunofluorescence (IF), and transmission electron microscopy (TEM). This allows for evaluation of the presence of immunoglobulins and complement components (IF), and the presence of glomerular electron-dense deposits and the remodelling of the glomerular basement membrane (TEM). This standard has not yet been established in diagnosing and classifying glomerular disease in dogs [130]. Likewise in this study the poor quality of the archived FFPE tissue prevented further ultrastructural characterisation of the glomerular changes in exudative GN via electron microscopy.

The present study contained a higher proportion of male compared to female dogs, which has been previously described in the literature [139-141]. This overrepresentation of males is thought to be linked to increased outdoor activity and canine male specific behaviour including sniffing and licking of urine, which likely promotes dog-to-dog transmission [90]. Higher temperatures and higher rainfall in April and November with a peak in late summer/early autumn correlated with the occurrence of cases in this study. In accordance with previous reports, puppies were overrepresented and a large range of breeds were affected [90, 115, 136]. Overall, exudative GN was not associated with any sex, age, time of year or particular breed, when compared to these parameters in the broader population of dogs with leptospirosis in Switzerland during the same study period [90].

Antemortem diagnosis of leptospirosis generally relies on the detection of high serum antibody titres using MAT, which detects serogroups and serovars. In acute fatal leptospirosis this method might fail to provide a positive result due to lack of time for an animal to develop an increased antibody titre, thus making it difficult for the clinician to confirm their clinical diagnostic suspicion. Pathologists face similar challenges if leptospirosis manifests in an unusual form as presented in this study which ultimately expands our clinicopathological picture of this disease.

Retrospective studies by their nature can be limited by the sample material and data available. The main limitation of this study was that it was not possible to determine the serovar(s) of the causative leptospires. Available blood samples did not yield a detectable increase in antibodies most of the time due to the acute nature of the infection. Additional molecular based methods to identify *Leptospira* sp. were limited due to the sampled tissue being formalin fixed and the presumed presence of amplification inhibitors. The high number of highly antigenically variable serovars of this organism and the fact that antibodies for IHC are not commercially available additionally hindered their identification. Future studies that include unfixed material for more detailed molecular analysis and sampling that allows complementary imaging (IF, TEM) are needed to further classify the pathogenesis of leptospiral exudative GN.

Conclusion

We described and associated exudative GN, a rarely reported glomerular pathology, with *Leptospira* and suspect that this lesion is caused by a species other than *L. interrogans*. Exudative GN was not associated with any specific age, breed, sex and season and clinical signs and pathology in tissues other than glomeruli was similar to what has been reported in the literature in cases of leptospirosis without GN. It is thus important for the clinician to consider that leptospirosis can manifest as acute exudative GN and treat accordingly, especially in cases when antibody levels have not yet risen, and the MAT remains negative.

IHC is the most sensitive tool to detect acute leptospirosis in FFPE material and its use can be complemented by qPCR. This study has highlighted the necessity to use multiple diagnostic tests and to target multiple serovars, especially when the ability to confirm leptospiral infection is restricted by limited availability of fixed material.

General Discussion

This thesis highlights the importance of innovative research on diseases and pathogens that have been known for a long time, like *Leptospira*. The objectives of the study were twofold: (i) to explore the incidence of *Leptospira* infection, especially the newly introduced *Leptospira* species *L. tipperaryensis,* in native and invasive species of shrews in Ireland and in turn to examine any possible negative effect this may have had on the native shrew population, and (ii) to investigate the unusual manifestation of exudative GN in dogs and its potential association with leptospirosis.

For the shrew study qPCR on fresh frozen renal tissue using specific primers for *L. tipperaryensis* and more general primers for pathogenic *Leptospira* species targeting outer membrane lipoprotein LipL32 did not detect any leptospiral DNA. The results of IHC supported these findings, where none leptospiral carrier was detected within the shrew population in Ireland in contrast to the control group sampled in France that had significant numbers of carrier animals.

Interestingly, significantly more GWTS were carriers of leptospirosis. A recent study investigating zoonotic pathogens in white-toothed shrews, i.e. bicoloured (C*rocidura leucodon*), greater (*Crocidura russula*) and lesser (*Crocidura suaveolens*) white-toothed shrews in Germany also detected the highest prevalence in GWTS compared to the other shrew species [105]. GWTS are considered range-expanding invaders [105, 106], which implies that they benefit from their behavioural flexibility during the invasion process, as they can expand their territory and even modify their ecological niche by claiming new food sources, shelters, or habitats. They may therefore have a higher exposure to *Leptospira* infection resulting in a higher incidence compared to other small mammalian species. The high prevalence of *Leptospira* in the urine of GWTS in 2013 could not be confirmed in this study. The original study cultured *Leptospira* from urinary samples, which could not be replicated in this thesis as urine samples were not available. It must be assumed that shrews in the 2013 study were shedding the pathogen, but that *Leptospira* organisms were not abundantly replicating in their renal tissue (as for instance in acute diseases) and thus we were not able to detect the spirochaetes with IHC. Furthermore, carrier status was significantly associated with more frequent and more severe renal inflammation, and the low inflammatory index in shrews sampled in Ireland is compatible with this finding. These results indicate that leptospirosis is not prevalent in pygmy shrews in Ireland and thus not contributing to the decline in their population. In line

with these conclusions are recent studies that suggest diet competition as the driving force behind the shift in habitat and local extinction of the pygmy shrews [85].

The study addressing the second objective of this thesis detected leptospiral antigens and nucleic acids in 74 % (37/50) of dogs that presented with exudative GN, confirming an association between these diseases. A similar detection rate of 73 % (36/49) was observed in cases that showed classic leptospiral lesions, highlighting the difficulties in diagnosing lesions retrospectively. Two different antibodies were used as part of the IHC protocol: one against LipL32 an outer membrane lipoprotein that is expressed by all pathogenic *Leptospira* species and one targeting OMV2177 antigen an LPS of *L. interrogans* serovar Copenhageni. The lower detection rate of OMV2177 in cases of exudative GN may indicate that the causative serovar is different from *L. interrogans* serovar Copenhageni (and antigenically related serovars). This suggestion is supported by the decrease of cases of exudative GN cases after the switch to multivalent vaccination in Switzerland that included more serovars, including Copenhageni.

Detecting *Leptospira* in the context of the studies within this thesis was often challenging and mirrors difficulties in detecting this bacterial species in general [53, 142]. Due to the transient nature of leptospiraemia, the agent is not continuously detectable in blood. The culture of *Leptospira* is a highly specific method of detection, requiring the absence of antibiotic treatment, fresh (non-autolytic) tissue and in the case of urine samples a suitable pH. Immunohistochemistry is a useful tool for detecting *Leptospira* spp. in necropsy cases or cases in which tissue is not suitable for culture, and when organisms are sufficiently plentiful to be visualised. Conventional and quantitative PCR-based assays provide highly sensitive and quick results when adequate testing material is provided. In contrast to those direct methods, the gold standard in clinical settings remains the MAT, which detects measurable increases in antibody levels. However, acute disease could be missed when using this approach, as antibody titres require 7 to 10 days to reach detectable concentrations. In summary, the time point of the disease, the available sample material and its condition, and the availability of tests (which might be limited for instance in developing countries) need to be taken into account when deciding which test or panel of tests to run. It must also be remembered that a negative result cannot necessarily completely outrule infection.

The outer membrane lipoprotein LipL32 was targeted in the studies of this thesis both in terms of its antigenic (IHC) and genomic (qPCR) structure. LipL32 is considered a virulence factor that is not present in non-pathogenic species, making it a crucial target for studying

pathogenic strains in general [108, 143]. This outer membrane lipoprotein is also the most widely used genetic marker for *Leptospira* detection, with 48 % of current studies detecting this sequence [111]. Subsequent steps to further identify leptospiral species would require additional targets like rrs2 (16S rRNA), secY (pre-protein translocase secY protein), or LipL41 (outer membrane lipoprotein LipL41) [144]. In this thesis IHC using antibody to detect LipL32 provided the most reliable overall detection. Nevertheless, traditional and quantitative PCR, especially of fresh tissue, remain established and robust methods of detecting leptospires [109, 120, 121].

This thesis provides new insights into the ever-evolving pathobiology of *Leptospira* given their demonstrated association with rarely described renal lesions – exudative GN – in dogs. A plausible explanation for the increased emergence of this lesion could be found in the changing epidemiology of leptospiral serovars, or even potential new strains. That this lesion has only been observed in Switzerland and possibly northern Italy [131] is of particular interest. If regional factors (like geography and climate), travel behaviour, or the lack of awareness of this specific pathology are the reason for this, its local distribution needs to be further explored. In addition, the dynamic of the newly discovered and introduced species *L. tipperaryensis* was re-evaluated after its first discovery in 2013 in Ireland [38]. However, the findings of this thesis do not confirm the establishment of this leptospiral species or a general significant incidence of any *Leptospira* species in shrews in Ireland. The previously high prevalence in GWTS reported [38] was not observed in this thesis. Possible explanations are likely to be found in host-serovar interactions or the availability of diagnostic tests such as culture.

Retrospective studies like those in this thesis have certain limitations: particularly in terms of the availability of suitable sample material. This thesis mostly relied on FFPE material, and throughout DNA extraction from FFPE tissue proved challenging and mostly unsatisfactory, even though the successful use of FFPE tissue for pathogen confirmation by qPCR has been described [128, 145]. Some FFPE blocks were stored for decades (the oldest dog sample was from 1996), which likely contributed to the limited use of qPCR for analysis. Our problem analysis identified poor DNA yield, amplification inhibitors, and possible contamination as the main difficulties with the extracted FFPE samples. Problem solving strategies like different extraction or molecular methods, or the clean-up of samples did not yield any additionally positive results. Fresh tissue was available for the shrew study, but unfortunately not for all of

the shrews sampled in Ireland and no fresh tissue was available for the shrews sampled in France. The availability of such tissues could have enhanced the detection rate of leptospiral genome.

Studies that rely on existing data have limited control over the quality of this information, which may contain errors or uncertainties in its analysis. Given the dogs in this study were examined by a multitude of clinicians and pathologists, there are some inconsistencies in the data and tissues collected. Retrospective studies are prone to reverse causality, making it difficult to establish the correct sequence of events and clearly determine cause and effect. Although no cases of exudative GN were observed in cases that were not suspected to have leptospirosis, certain diagnostic biases cannot be completely excluded, and additional factors like immune-mediated processes unrelated to leptospirosis might contribute to the development of this specific lesion. Despite these drawbacks, retrospective studies are still valuable and can provide important insights, especially in situations where conducting prospective studies is neither feasible nor ethical. Retrospective research can generate hypotheses for further investigation, offer valuable real-world insights into pathogenesis, and encourage collaborations and information exchange among researchers in the area.

This thesis will have an impact on our understanding of a number of aspects of leptospirosis. The identification and description of the previously unknown renal changes that we associated with *Leptospira* infection is an important step in defining this diagnosis. This is important for both the monitoring of the epidemiology of leptospiral serovars, and to get more insights into the pathogenesis of leptospirosis, which is especially important as this disease is a zoonosis. The identification and description of previously unrecognised renal lesions may be attributed to the circulation of a novel serovar, and as mentioned above our findings suggest that the causative serovar differs from *Leptospira interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni. Cases of leptospirosis and exudative GN have decreased since vaccination protocols have been updated from the previous bivalent vaccination (Icterohaemorrhagiae and Canicola) to the current four serogroups (Icterohaemorrhagiae, Canicola, Australis, Grippotyphosa) which cover the most common serovars in Europe: Australis, Bratislava, Copenhageni, Canicola, Grippotyphosa, and Icterohaemorrhagiae [115]. This practical response to research findings highlights the importance of ongoing monitoring and identification of circulating and novel pathogens, especially when increased travel behaviour and climate change are potentially increasing host exposure to new serovars. The

ongoing surveillance of the canine population and the regular re-evaluation of the effectiveness of current vaccination schemes is thus highly recommended.

The same principle applies to newly identified serovars, both in wildlife and domestic species. It is crucial to thoroughly investigate the pathological capabilities of new *Leptospira* species in order to monitor their potential to cause clinical disease. Evaluating the disease impact of leptospiral serovars on various species, including pets, livestock, humans and wildlife, is of utmost importance. Leptospiral serovars are adapted to different species, and their ability to cause disease depends on the species infected. A thorough and regular assessment and screening for *Leptospira* allows for a better understanding and management of the associated risks posed by emerging serovars, and provides opportunities to adapt the management of those risks. For example, the modified vaccination protocol described above resulted in decreased canine leptospirosis cases, which ultimately decreased the infectious pressure on other animals and on humans [53, 115].

Thorough and regular monitoring of wildlife health is crucial for several reasons including the One Health paradigm [146]. In the context of this thesis wildlife health monitoring has contributed to our understanding and analysis of the complexity of the leptospiral genus. Ongoing genetic analysis continuously redefines the relatedness of *Leptospira* serovars, and has led to the discovery of new species and serovars. The rapid and severe decline of the native pygmy shrew population in Ireland associated with the introduction and increased distribution of invasive GWTS [37, 85] needed to be investigated for the potential co-introduction of an infectious agent, such as *Leptospira* spp.. Leptospirosis is also a major zoonosis that can, if not recognised early, develop into a severe and fatal disease. The monitoring of wildlife and domestic species can identify carriers and changes in seroprevalence that allow for a better understanding and management of the associated risks and potential zoonotic spill-overs. The results of this study do not indicate that shrews in Ireland (either native or invasive) are carriers of *Leptospira* and thus contribute little to the maintenance of infection within the environment. Overall, monitoring wildlife health is vital for the conservation of species, the detection and control of diseases, and the protection of human health.

Future studies that aim to optimise the elimination of amplification inhibitors in FFPE samples without losing nucleic acid content are needed. This would allow further explorative investigations like the determination of serovars, which might then be linked to specific lesions.

A prospective collection of canine cases to sample suitable material for TEM could help to further understand the underlying pathogenesis of exudative GN. The opportunity to culture isolates would provide a valuable resource for identifying the causative species and analysing its genetic and virulence factors. Regarding shrews, an extended monitoring of different (wild and domestic) species for *L. tipperaryensis* could offer insights into its origin and distribution. Additional in vivo studies could be used to investigate whether *L. tipperaryensis* promotes primarily a 'carrier' immune response, i.e. no clinical disease, in shrews or maybe a more unexpected, severe inflammatory reaction as seen in incidental hosts.

This thesis provides new and important insights into the aetiopathogenesis of leptospirosis. Examining shrews sampled in Ireland provided two valuable pieces of information. Firstly, that shrew populations in Ireland are not significant carriers of *Leptospira* and are thus not a source of infection for other animals or humans, and secondly that *Leptospira* infection has not contributed to the regional decline or extinction of the native pygmy shrew. This thesis is also the first to define canine exudative GN, an unusual and uncommon lesion, and link it with leptospiral infection. Multiple methods of detecting *Leptospira* spp. were utilised throughout this thesis in order to optimally detect this fastidious pathogen.

Appendix 1 – References

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Appendix 2 – Chapter 2

Appendix Table A1. NanoDrop™ Thermo Scientific™ measurements of nucleic acid concentration within FFPE shrew samples.

Shrew ID	Country	Sample Year	Species	Tissue	Nucleic Acid (ng/uL)	A260/A280
R156	Ireland	2013	GWTS	FFPE	1226.13	1.997
R159	Ireland	2013	GWTS	FFPE	732.38	2.037
R158	Ireland	2013	GWTS	FFPE	609.32	2.086
R161	Ireland	2013	GWTS	FFPE	536.52	2.056
R157	Ireland	2013	GWTS	FFPE	464.07	2.000
R164	Ireland	2013	GWTS	FFPE	453.66	2.037
R160	Ireland	2013	GWTS	FFPE	416.74	2.037
R162	Ireland	2013	GWTS	FFPE	359.80	2.070
R163	Ireland	2013	GWTS	FFPE	330.00	2.003
R165	Ireland	2013	GWTS	FFPE	294.36	2.022

Appendix Table A2. NanoDrop™ Thermo Scientific™ measurements of nucleic acid concentration within fresh shrew samples.

Shrew ID	Country	Sample Year	Species	Tissue	Nucleic Acid (ng/uL)	A260/A280
E50	Ireland	2018	GWTS	Fresh frozen	433.79	2.013
E47	Ireland	2018	GWTS	Fresh frozen	384.15	2.022
E48	Ireland	2018	GWTS	Fresh frozen	375.78	2.008
E46	Ireland	2018	GWTS	Fresh frozen	369.35	2.000
E45	Ireland	2018	GWTS	Fresh frozen	359.75	2.004
E51	Ireland	2018	GWTS	Fresh frozen	323.31	2.037
E54	Ireland	2018	GWTS	Fresh frozen	311.13	2.019
E58	Ireland	2018	Pygmy	Fresh frozen	307.27	1.972
E73	Ireland	2018	GWTS	Fresh frozen	302.30	2.093
S10	Ireland	2017	GWTS	Fresh frozen	299.08	1.968
E60	Ireland	2018	GWTS	Fresh frozen	296.98	2.008
E55	Ireland	2018	GWTS	Fresh frozen	290.61	2.007
E52	Ireland	2018	GWTS	Fresh frozen	288.57	2.033
E71	Ireland	2018	GWTS	Fresh frozen	281.41	2.049

W73	Ireland	2018	GWTS	Fresh frozen	279.14	1.996
E62	Ireland	2018	GWTS	Fresh frozen	272.00	2.074
E53	Ireland	2018	GWTS	Fresh frozen	268.48	2.016
E65	Ireland	2018	GWTS	Fresh frozen	268.40	2.047
E57	Ireland	2018	Pygmy	Fresh frozen	262.63	1.969
E84	Ireland	2018	GWTS	Fresh frozen	253.05	1.975
E64	Ireland	2018	GWTS	Fresh frozen	251.67	2.043
W62	Ireland	2018	GWTS	Fresh frozen	250.21	2.027
W75	Ireland	2018	GWTS	Fresh frozen	242.14	2.018
S52	Ireland	2018	GWTS	Fresh frozen	241.32	1.981
E59	Ireland	2018	GWTS	Fresh frozen	241.15	2.001
E85	Ireland	2018	GWTS	Fresh frozen	241.08	2.006
E70	Ireland	2018	GWTS	Fresh frozen	240.58	2.061
E83	Ireland	2018	GWTS	Fresh frozen	233.77	2.022
W68	Ireland	2018	GWTS	Fresh frozen	231.92	2.048
W71	Ireland	2018	Pygmy	Fresh frozen	229.37	1.979
W69	Ireland	2018	GWTS	Fresh frozen	224.26	2.000
E63	Ireland	2018	GWTS	Fresh frozen	223.79	2.008
W74	Ireland	2018	Pygmy	Fresh frozen	217.17	1.969
W67	Ireland	2018	GWTS	Fresh frozen	216.74	2.029
W72	Ireland	2018	GWTS	Fresh frozen	216.41	1.993
E76	Ireland	2018	GWTS	Fresh frozen	216.30	2.004
S11	Ireland	2017	GWTS	Fresh frozen	216.13	1.990
W01	Ireland	2017	GWTS	Fresh frozen	212.95	1.987
S17	Ireland	2017	Pygmy	Fresh frozen	212.45	1.957
S42	Ireland	2018	GWTS	Fresh frozen	209.96	1.952
W23	Ireland	2017	GWTS	Fresh frozen	209.84	1.994
W64	Ireland	2018	GWTS	Fresh frozen	206.57	2.001
W76	Ireland	2018	GWTS	Fresh frozen	205.19	1.933
S14	Ireland	2017	Pygmy	Fresh frozen	204.39	1.939
S56	Ireland	2018	GWTS	Fresh frozen	203.89	1.963
W79	Ireland	2018	Pygmy	Fresh frozen	202.22	1.934
W29	Ireland	2017	GWTS	Fresh frozen	201.70	2.016
E74	Ireland	2018	GWTS	Fresh frozen	201.06	2.011
S06	Ireland	2017	GWTS	Fresh frozen	196.93	1.954

S12	Ireland	2017	Pygmy	Fresh frozen	196.85	1.967
S37	Ireland	2017	GWTS	Fresh frozen	196.37	1.980
S48	Ireland	2018	GWTS	Fresh frozen	194.85	1.960
W70	Ireland	2018	Pygmy	Fresh frozen	192.08	1.985
S13	Ireland	2017	Pygmy	Fresh frozen	187.20	1.922
S21	Ireland	2017	Pygmy	Fresh frozen	186.54	1.952
S55	Ireland	2018	GWTS	Fresh frozen	184.75	1.957
W65	Ireland	2018	GWTS	Fresh frozen	183.18	2.004
S07	Ireland	2017	GWTS	Fresh frozen	182.62	1.972
W22	Ireland	2017	GWTS	Fresh frozen	180.90	1.997
S60	Ireland	2018	GWTS	Fresh frozen	180.46	1.966
S57	Ireland	2018	GWTS	Fresh frozen	179.44	1.969
S65	Ireland	2018	GWTS	Fresh frozen	178.75	2.011
S15	Ireland	2017	Pygmy	Fresh frozen	177.36	1.935
E82	Ireland	2018	Pygmy	Fresh frozen	176.25	1.924
S40	Ireland	2017	GWTS	Fresh frozen	174.48	1.998
W15	Ireland	2017	Pygmy	Fresh frozen	172.88	1.994
E61	Ireland	2018	Pygmy	Fresh frozen	172.59	1.931
W63	Ireland	2018	Pygmy	Fresh frozen	169.61	1.947
W06	Ireland	2017	GWTS	Fresh frozen	169.55	1.961
S39	Ireland	2017	GWTS	Fresh frozen	168.85	1.965
W16	Ireland	2017	Pygmy	Fresh frozen	168.34	2.015
S47	Ireland	2018	GWTS	Fresh frozen	167.55	1.916
W80	Ireland	2018	Pygmy	Fresh frozen	166.61	1.963
S20	Ireland	2017	Pygmy	Fresh frozen	165.40	1.939
S31	Ireland	2017	GWTS	Fresh frozen	165.23	2.003
S27	Ireland	2017	Pygmy	Fresh frozen	163.65	1.931
E56	Ireland	2018	Pygmy	Fresh frozen	163.28	1.945
S43	Ireland	2018	GWTS	Fresh frozen	162.96	1.977
W78	Ireland	2018	Pygmy	Fresh frozen	162.48	1.969
S68	Ireland	2018	Pygmy	Fresh frozen	160.25	1.983
S16	Ireland	2017	Pygmy	Fresh frozen	159.12	1.934
S02	Ireland	2017	GWTS	Fresh frozen	158.98	1.972
S44	Ireland	2018	GWTS	Fresh frozen	157.34	2.018
S59	Ireland	2018	GWTS	Fresh frozen	157.16	1.961

S04	Ireland	2017	GWTS	Fresh frozen	156.99	1.995
W77	Ireland	2018	Pygmy	Fresh frozen	156.51	1.971
W04	Ireland	2017	GWTS	Fresh frozen	151.55	1.944
E79	Ireland	2018	Pygmy	Fresh frozen	150.30	1.936
E81	Ireland	2018	Pygmy	Fresh frozen	150.00	1.940
W02	Ireland	2017	GWTS	Fresh frozen	147.58	2.018
S36	Ireland	2017	GWTS	Fresh frozen	144.68	1.989
S61	Ireland	2018	GWTS	Fresh frozen	144.18	2.004
W03	Ireland	2017	GWTS	Fresh frozen	143.64	1.946
W09	Ireland	2017	GWTS	Fresh frozen	143.51	1.987
S28	Ireland	2017	Pygmy	Fresh frozen	143.50	1.940
S70	Ireland	2018	Pygmy	Fresh frozen	143.23	1.938
S41	Ireland	2018	Pygmy	Fresh frozen	142.99	1.929
S19	Ireland	2017	Pygmy	Fresh frozen	141.98	1.939
S54	Ireland	2018	GWTS	Fresh frozen	141.80	1.942
S09	Ireland	2017	GWTS	Fresh frozen	141.35	1.981
S38	Ireland	2017	GWTS	Fresh frozen	140.73	2.014
S03	Ireland	2017	GWTS	Fresh frozen	140.52	1.974
S33	Ireland	2017	GWTS	Fresh frozen	138.97	1.989
W34	Ireland	2017	Pygmy	Fresh frozen	137.38	1.902
S01	Ireland	2017	Pygmy	Fresh frozen	136.93	1.910
S08	Ireland	2017	GWTS	Fresh frozen	136.07	1.952
W07	Ireland	2017	GWTS	Fresh frozen	135.63	1.978
S53	Ireland	2018	GWTS	Fresh frozen	135.52	1.970
E67	Ireland	2018	Pygmy	Fresh frozen	135.19	1.992
S22	Ireland	2017	Pygmy	Fresh frozen	134.97	1.903
W20	Ireland	2017	GWTS	Fresh frozen	134.83	2.002
W66	Ireland	2018	GWTS	Fresh frozen	132.69	2.090
S51	Ireland	2018	Pygmy	Fresh frozen	130.43	1.886
S18	Ireland	2017	Pygmy	Fresh frozen	130.27	1.928
S75	Ireland	2018	Pygmy	Fresh frozen	129.11	1.908
W26	Ireland	2017	GWTS	Fresh frozen	129.02	2.043
S45	Ireland	2018	Pygmy	Fresh frozen	126.78	1.981
W18	Ireland	2017	Pygmy	Fresh frozen	126.63	1.989
E75	Ireland	2018	GWTS	Fresh frozen	125.76	2.019

S50	Ireland	2018	Pygmy	Fresh frozen	125.40	1.880
S30	Ireland	2017	Pygmy	Fresh frozen	125.29	1.947
S05	Ireland	2017	GWTS	Fresh frozen	125.10	1.962
W12	Ireland	2017	Pygmy	Fresh frozen	125.05	1.969
W27	Ireland	2017	GWTS	Fresh frozen	124.98	2.026
S32	Ireland	2017	GWTS	Fresh frozen	124.59	1.984
S49	Ireland	2018	Pygmy	Fresh frozen	124.35	1.898
S64	Ireland	2018	Pygmy	Fresh frozen	123.91	1.980
W28	Ireland	2017	Pygmy	Fresh frozen	122.56	2.073
W21	Ireland	2017	Pygmy	Fresh frozen	122.01	1.964
S35	Ireland	2017	GWTS	Fresh frozen	117.58	1.975
E66	Ireland	2018	Pygmy	Fresh frozen	117.04	1.967
S62	Ireland	2018	GWTS	Fresh frozen	114.62	2.009
W37	Ireland	2017	Pygmy	Fresh frozen	114.44	1.936
W60	Ireland	2018	Pygmy	Fresh frozen	114.42	1.905
S69	Ireland	2018	Pygmy	Fresh frozen	113.43	1.946
S46	Ireland	2018	Pygmy	Fresh frozen	110.89	1.897
S34	Ireland	2017	GWTS	Fresh frozen	110.79	1.982
S26	Ireland	2017	Pygmy	Fresh frozen	110.43	1.935
W10	Ireland	2017	GWTS	Fresh frozen	110.06	2.029
W13	Ireland	2017	Pygmy	Fresh frozen	108.79	2.045
E72	Ireland	2018	GWTS	Fresh frozen	104.95	2.027
S63	Ireland	2018	Pygmy	Fresh frozen	103.19	1.927
E80	Ireland	2018	Pygmy	Fresh frozen	102.90	1.919
S29	Ireland	2017	Pygmy	Fresh frozen	102.24	1.942
W36	Ireland	2017	Pygmy	Fresh frozen	100.53	1.937
S74	Ireland	2018	Pygmy	Fresh frozen	99.64	1.964
S58	Ireland	2018	GWTS	Fresh frozen	97.20	1.970
W17	Ireland	2017	Pygmy	Fresh frozen	96.39	2.019
S73	Ireland	2018	Pygmy	Fresh frozen	95.26	1.920
E86	Ireland	2018	Pygmy	Fresh frozen	95.20	1.948
E69	Ireland	2018	Pygmy	Fresh frozen	91.08	1.980
W19	Ireland	2017	GWTS	Fresh frozen	89.65	2.024
W24	Ireland	2017	Pygmy	Fresh frozen	88.97	1.988
W11	Ireland	2017	Pygmy	Fresh frozen	87.91	1.975

S67	Ireland	2018	Pygmy	Fresh frozen	87.17	2.011
W05	Ireland	2017	GWTS	Fresh frozen	87.07	1.958
W30	Ireland	2017	GWTS	Fresh frozen	79.75	2.088
W61	Ireland	2018	Pygmy	Fresh frozen	77.51	1.875
W33	Ireland	2017	Pygmy	Fresh frozen	75.56	1.953
W38	Ireland	2017	Pygmy	Fresh frozen	75.53	1.914
W08	Ireland	2017	GWTS	Fresh frozen	74.95	1.970
W25	Ireland	2017	GWTS	Fresh frozen	68.77	1.951
E68	Ireland	2018	Pygmy	Fresh frozen	66.44	1.969
W35	Ireland	2017	Pygmy	Fresh frozen	57.64	1.879
W59	Ireland	2018	Pygmy	Fresh frozen	56.64	1.893
W14	Ireland	2017	Pygmy	Fresh frozen	49.26	1.991
W31	Ireland	2017	GWTS	Fresh frozen	39.94	2.061
W32	Ireland	2017	Pygmy	Fresh frozen	34.42	1.962

Appendix Table A3. Histopathological changes according to species, country, and carrier status.

	Total	Irish PS	Irish GWTS	French PS	French GWTS	Carrier
Main findings						
Tubular degeneration	1.0 % (3/311)	0.0 % (0/96)	1.5 % (2/134)	0.0 % (0/41)	2.5 % (1/40)*	1 (3.6 %)
Neutrophilic nephritis	4.8 % (15/311)	3.1 % (3/96)	6.0 % (8/134)	0.0 % (0/41)	10.0 % (4/40)	2 (7.1 %)
Chronic nephritis	20.6 % (64/311)	14.6 % (12/96)	13.4 % (18/134)	12.2 % (5/41)	72.5 % (29/40)	23 (82.1 %)
Unspecific findings						
Protein casts	20 (6.4 %)	0 (0.0 %)	14 (10.4 %)	3 (7.3 %)	3 (7.5 %)	2 (7.1 %)
Glomerular changes	2 (0.6 %)	1 (1.0 %)	1 (0.7 %)	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)
Haemorrhage, interstitial	1 (0.3 %)	0 (0.0 %)	1 (0.7 %)	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)
Hydronephrosis	1 (0.3 %)	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	1 (2.5 %)	0 (0.0 %)
Total number	311	96	134	41	40	28

* This animal was also affected by neutrophilic interstitial nephritis.

Appendix 3 – Chapter 3

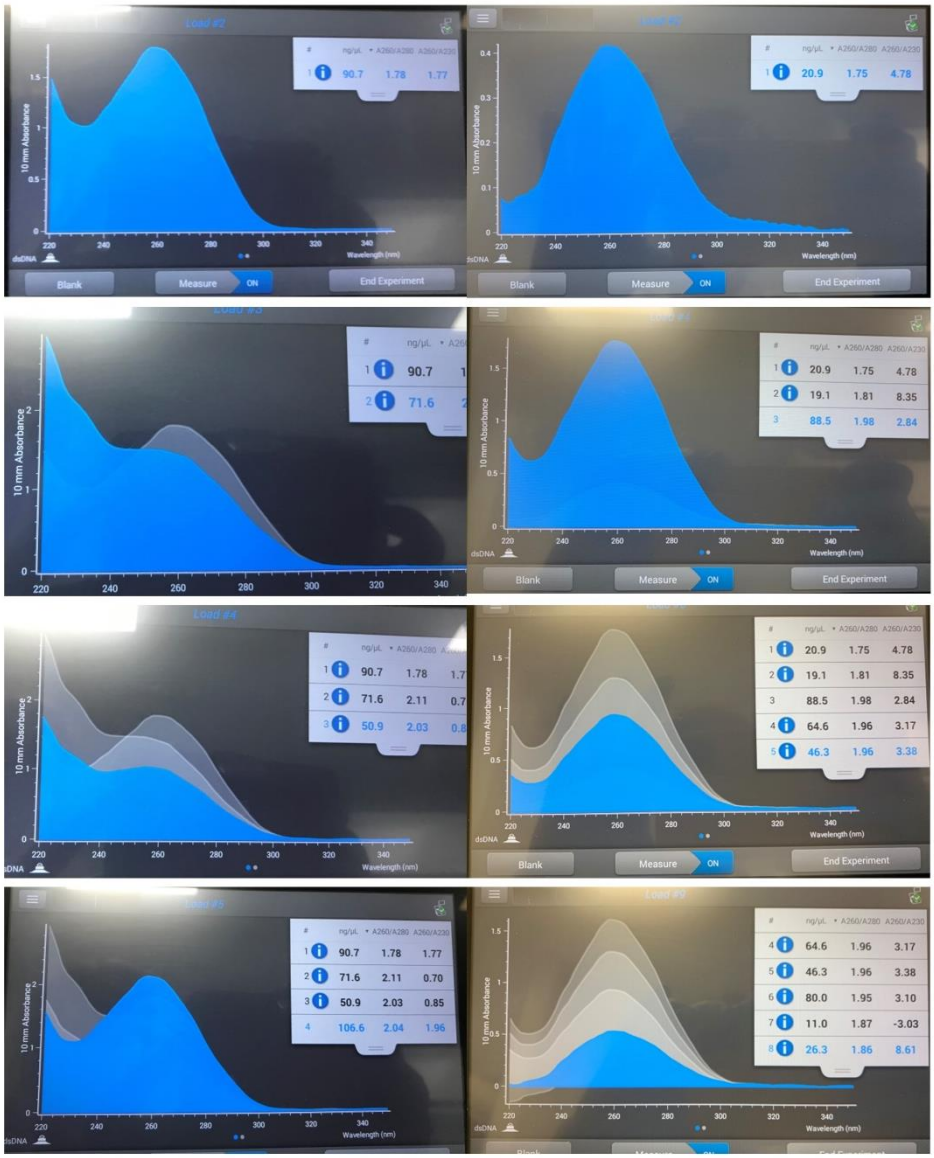
Appendix Table A1. NanoDrop™ Thermo Scientific™ measurements of nucleic acid concentration within the canine samples.

ID	Nucleic Acid(ng/uL)	A260/A280
S00-0019.2	1226.13	2.00
S01-1731.5	797.01	1.97
S05-1338.1	767.38	2.05
S00-1289.4	732.38	2.04
S97-3669.6	701.90	2.04
S07-1373.1	698.22	2.07
S06-1142.3	650.24	2.09
S03-1252.3	641.68	2.01
S08-1430.3	636.33	2.08
S00-0921.2	609.32	2.09
S07-0885.1	587.78	2.11
S05-1348.1	587.03	2.09
S03-1253.1	586.97	2.07
S10-1493.1	585.78	2.07
S02-1326.3	585.08	2.06
S07-1487.1	573.24	2.04
S06-1142.2	569.05	2.11
S02-1361.3	568.23	2.05
S00-1482.3	536.52	2.06
S07-1103.1	527.08	2.07
S96-2053.3	527.00	2.06
S96-2189.1	515.51	2.05
S04-1464.2	512.73	2.08
S02-0831.4	495.61	1.98
S14-2641.2	487.74	2.00
S02-0979.3	487.01	2.05
S00-0657.3	464.07	2.00
S01-0789.1	453.66	2.04
S02-0885.3	445.31	2.03
S00-1352.4	416.74	2.04

S10-0546.1	409.95	2.09
S05-1575.2	407.79	2.07
S02-1597.2	401.80	2.04
S04-1342.1	375.88	2.08
S15-1103.1	373.29	2.06
S07-1104.1	364.39	2.08
S00-1600.5	359.80	2.07
S01-0285.2	330.00	2.00
S97-3400.4	317.10	2.02
S10-1425.1	316.55	2.08
S14-0206.4	306.45	1.73
S01-1455.2	294.36	2.02
S04-1629.4	279.35	2.08
S13-1397.1	277.38	2.03
S05-1356.1	275.26	2.10
S10-0976.8	272.66	2.06
S04-1616.1	259.13	2.08
S12-1056.1	254.30	2.07
S07-2104.1	245.56	2.11
H09-3100.4	241.60	1.97
S09-1170.1	239.75	2.08
S01-1656.1	238.56	2.08
S04-1112.3	233.78	2.01
S04-1360.6	206.80	2.09
S07-1365.1	204.68	2.12
S97-3542.1	192.39	2.05
S03-1944.1	181.74	2.10
S01-1834.4	181.51	2.00
S99-1772.6	155.92	2.00
S98-2019.1	155.70	1.98
S98-1891.5	154.45	2.01
S05-1528.2	149.41	2.09
S15-0742.5	147.99	2.05
S99-1828.3	145.83	2.03
H09-2464.2	141.04	1.98

S99-1317.4	123.76	2.00
S99-1009.1	122.76	2.03
S99-1674.9	122.45	2.01
S98-1470.8	117.52	1.98
S98-1261.5	109.71	2.03
S08-1264.1	99.22	2.22
S99-1910.4	92.44	2.03
S10-1066.4	90.17	2.13
H08-0397.2	84.36	2.03
H08-2169.1	81.98	2.01
H08-3067.2	56.82	2.26
S99-1399.8	49.38	2.11
S08-1291.1	28.31	2.68
S99-1351.3	8.64	6.91
S99-1265.3	4.91	-0.64

Appendix Figure 1. Absorption curve of extraction with deparaffinization solution and xylene



Nano Drop absorption curves; same sample with deparaffinisation extraction on the left side and xylene extraction on the right side.

Appendix 4 – Case Log

Necropsies

Necropsy Date	Necropsy Number	Species family	Breed	Main findings
06.01.20	N1/20	Bovine	Limousin	Dermatosparaxis
07.01.20	N4/20	Canine	Lurcher	Lymphoma
08.01.20	N8/20	Bovine	Charolais	Osteomyelitis
09.01.20	N12/20	Caprine	NA	Johne's disease
13.01.20	N17/20	Canine	Dachshund	Pancreatitis (Diabetes)
13.01.20	N19/20	Canine	Yorkshire Terrier	Bite trauma
14.01.20	N21/20	Zoo	Oryx	Necrotising-haemorrhagic enteritis
16.01.20	N23/20	Canine	German Shepherd Dog (GSD)	Lymphosarcoma
16.01.20	N24/20	Canine	Jack Russel Terrier (JRT)	Pulmonary thrombosis
20.01.20	N27/20	Canine	Golden Retriever	Suggestive of cardiovascular collapse (acute heart failure)
21.01.20	N30/20	Bovine	Limousin	Intestinal stenosis and hypoplasia, born dead
27.01.20	N38/20	WILD	Grey seal	Suppurative cellulitis
27.01.20	N39/20	WILD	Grey seal	Emaciation
28.01.20	N42/20	Reptilia	Indigo snake	ulcerative to necrotising gastroenteritis with marked chronic hepatic fibrosis
29.01.20	N45/20	Feline	Domestic short-haired cat (DSH)	Hyperthyroidism and follicular cell adenomas
30.01.20	N47/20	Caprine	NA	Johne's disease
31.01.20	N51/20	Bovine	Limousin	Severe suppurative bronchopneumonia
03.02.20	N55/20	Ovine	NA	Abortion
03.02.20	N56/20	Ovine	NA	Foetal maceration
04.02.20	N58/20	Bovine	Jersey	*E.coli* enteritis
05.02.20	N61/20	WILD	Hedgehog	Choroiditis and meningitis

06.02.20	N64/20	Canine	Labrador	Carcinoma, potential thyroid adenocarcinoma (Liver, Lung, Spleen, LNN)
10.02.20	N69/20	Ovine	NA	Stillbirth w/o cause
10.02.20	N71/20	WILD	Grey seal	Pyothorax
11.02.20	N78/20	Feline	DSH	Liver tumour and diaphragmatic hernia
12.02.20	N80/20	Canine	Boxer	Chemodectoma
13.02.20	N83/20	WILD	Harbour seal	Septicaemia
13.02.20	N84/20	Feline	DSH	Hypertrophic cardiomyopathy
17.02.20	N89/20	WILD	Grey seal	Interstitial pneumonia
18.02.20	N93/20	Zoo	Meerkat	Suppurative meningoencephalitis and thrombosis
18.02.20	N95/20	LAB	Mouse	Septicaemia
20.02.20	N99/20	Zoo	Lizard	Obstipation
21.02.20	N102/20	LAB	Mouse	Bacteraemia
25.02.20	N109/20	Feline	DSH	Hypertrophic cardiomyopathy
25.02.20	N113/20	LAB	Mouse	Bacteraemia
26.02.20	N115/20	Bovine	NA	*Trueperella* abortion
28.02.20	N116/20	Canine	Crossbreed	Necrohaemorrhagic enterocolitis
02.03.20	N123/20	WILD	Grey seal	Emaciation
02.03.20	N124/20	WILD	Grey seal	Sepsis
04.03.20	N125/20	Canine	Portuguese water dog	Hypoadrenocorticism (Addison's disease)
05.03.20	N126/20	Bovine	Jersey	Bronchopneumonia
06.03.20	N128/20	Avian	Bulgarian	Presumptive seminoma
12.05.20	N157/20	Caprine	NA	Haired skin (mass): Angiomatosis/hemangiosarcoma
13.05.20	N156/20	Avian	Stork	Acute systemic infectious process
13.05.20	N158/20	Camelidae	Alpaca	Epi and myocarditis; hepatic necrosis
15.05.20	N160/20	Avian	Canary	Severe necrotising tracheitis
25.05.20	N166/20	Camelidae	Alpaca	Presumptive placental disorder with foetal malnutrition fat mobilisation & fatty liver
27.05.20	N168/20	Lagomorph	NA	RHDV-2
29.06.20	N200/20	Bovine	Holstein	Ceacal torsion and dilatation

01.07.20	N202/20	Canine	St Bernard	GDV
03.07.20	N206/20	Equine	Horse	Colon torsion
07.07.20	N207/20	Camelidae	Alpaca	Bilateral choanal atresia.
08.07.20	N208/20	Canine	Labrador	Systemic inflammatory condition such as septicaemia - reason unknown
08.07.20	N210/200	Feline	DSH	Parvo enteritis
09.07.20	N211/20	Canine	Dalmatian	Septicaemia (renal abscesses) and oedema seen in brain and heart
09.07.20	N212/20	Bovine	Limousin	Ulcerations in the oral cavity and pharynx, abomasitis, and necrotising enteritis and typhlitis - unknown reason
10.07.20	N215/20	WILD	Grey seal	Apnoea in anaesthesia
20.07.20	N228/20	Lagomorph	Rabbit	Pneumonia vs haemorrhages
21.07.20	N231/20	Ovine	NA	CNN
23.07.20	N232/20	Canine	Greyhound	Enteritis and pituitary tumour
24.07.20	N233/20	Canine	Labrador	Rib tumour - osteosarcoma
24.07.20	N234/20	Avian	Canary	NSF
11.08.20	N257/20	Feline	Maine Coon	Diaphragmatic/pleuroperitoneal hernia with herniation of stomach
11.08.20	N258/20	Avian	Swan	Proventriculitis & enteritis
12.08.20	N262/20	Bovine	Limousin	Lungworm pneumonia
13.08.20	N264/20	Canine	Chihuahua	Splenic lymphoma
08.09.20	N305/20	WILD	Red river hog	Marked chronic renal changes with presumptive uraemia
10.09.20	N306/20	Feline	DSH	Septicaemia (Beta-haemolytic *E. coli*)
11.09.20	N307/20	Avian	Lovebird	NSF
14.09.20	N312/20	Bovine	Holstein	Hardware disease
15.09.20	N318/20	Bovine	Friesian	Bacterial enteritis
18.09.20	N322/20	Avian	Flamingo	Coelomitis and effusion
22.09.20	N327/20	Bovine	Friesian	Fog fever
22.09.20	N328/20	Feline	BSH	Trauma
23.09.20	N329/20	Canine	Greyhound	Alabama Rot
24.09.20	N332/20	Canine	Yorkshire Terrier	Round-cell neoplasm (presumptive lymphocytic)

29.09.20	N341/20	Zoo	Chimpanzee	Dissecting myocardial fibrosis/ cardiomyopathy
12.10.20	N367/20	Zoo	Macaque	Cardiomyocyte necrosis
13.10.20	N368/20	Feline	DSH	Non-regenerative thrombocytopenia viral aetiology
19.10.20	N374/20	Equine	NA	Splenic lymphoma
20.10.20	N379/20	Zoo	Vulture	Likely septicaemia with vasculitis and inflammation in multiple organs
21.10.20	N381/20	Canine	Labrador	Rib: Neoplasia (suspect osteosarcoma)
21.10.20	N384/20	Lagomorph	NA	Hepatitis (RHDV)
22.10.20	N382/20	WILD	Harbour seal	Hepatic necrosis and meningitis
22.10.20	N383/20	WILD	Harbour seal	Interstitial lymphoplasmacytic pneumonia
23.10.20	N386/20	Canine	Terrier X	Pheochromocytoma
03.11.20	N394/20	Avian	Swan	Emaciation - *Echinuria uncinate*, *Proteus* and *E. coli* infection
03.11.20	N395/20	Avian	Cockatiel	Proventriculus with bacteria and cell debris
09.11.20	N406/20	WILD	Harbour seal	Stomach ulceration (*Anisakis*) and Emaciation
09.11.20	N407/20	WILD	Harbour seal	Encephalitis and pneumonia with presumptive septicaemia
12.11.20	N418/20	Canine	GSD	Presumptive cardiomyopathy and immune mediated disease
24.11.20	N433/20	Feline	DSH	Hypertrophic cardiomyopathy
25.11.20	N438/20	WILD	Grey seal	Bacterial bronchopneumonia and necrotising bacterial enterocolitis
30.11.20	N441/20	WILD	Grey seal	Abscess, bite wounds and presumptive septicaemia
30.11.20	N442/20	WILD	Harbour seal	Ulcerative gastritis and bronchopneumonia
01.12.20	N448/20	Canine	French Bulldog	Foreign body in pharynx
02.12.20	N450/20	Canine	Jack Russel Terrier	Adrenal: malignant pheochromocytoma.

	N469/20	Canine	Greyhound	GDV
	N473/20	Feline	DSH	Hypertrophic cardiomyopathy
19.01.21	N10/21	Zoo	Bush Dog	Septicaemia; phaeochromocytoma nodular hyperplasia
19.01.21	N13/21	Ovine	Texel	Unilateral bronchopneumonia
20.01.21	N15/21	Canine	Rottweiler	ITP
21.01.21	N16/21	Canine	Bavarian Scent Hound	Prematurity with systemic infection with vasculitis, thrombi, hepatic necrosis and pneumonia
22.01.21	N17/21	Zoo	Wallaby	Emaciation
03.02.21	N24/21	Canine	French Bulldog	sepsis
04.02.21	N25/21	WILD	Grey Seal	Leptospirosis
05.02.21	N28/21	WILD	Turtle dove	Pyogranulomatous inflammation consistent with yersiniosis
09.03.21	N64/21	WILD	Grey seal	Emaciation
12.03.21	N66/21	Zoo	Squirrel Monkey	Myocardial dissecting fibrosis
16.03.21	N67/21	WILD	Harbour deal	Bite wound with embolic spread and pneumonia
16.03.21	N68/21	WILD	Grey deal	Interstitial pneumonia - secondary to septicaemia
16.03.21	N71/21	Canine	Bichon Frise	IMPA
18.03.21	N72/21	Canine	Collie	Renal injury consistent with leptospirosis
29.03.21	N91/21	Canine	Golden Doodle	IMHA
12.04.21	N102/21	WILD	Grey seal	Pneumonia and melena
13.04.21	N104/21	WILD	Grey seal	Neck deformation
13.04.21	N105/21	WILD	Harbour seal	Emaciation
16.04.21	N108/21	Canine	Labrador X	Hemangiosarcoma
08.06.21	N177/21	Zoo	Ibis	Unclear
08.06.21	N180/21	Canine	Collie	Acute ischaemic/hypoxic injury
08.06.21	N181/21	Canine	Schnauzer	Malignant chemodectoma (aortic body carcinoma)
09.06.21	N184/21	Zoo	Macaque	Placentitis with sepsis

10.06.21	N186/21	Canine	Weimaraner	Endocarditis and pancreatic necrosis vs neoplasia
11.06.21	N188/21	Canine	French Bulldog	Systemic infection - no cause
11.06.21	N191/21	WILD	Grey seal	Icterus
28.06.21	N225/21	Lagomorph	NA	Cardiomyopathy with intracardial thrombus
28.06.21	N226/21	Porcine	Pig	Haemorrhagic urocystitis
29.06.21	N228/21	Canine	Yorkshire Terrier	Omphalitis (navel ill)
29.06.21	N229/21	Equine	Pony	Penile SCC
30.06.21	N230/21	Zoo	Gyrfalcon	NSF
30.06.21	N231/21	Bovine	Saler	Fibrinous peritonitis with abdominal abscesses & Enteritis
01.07.21	N232/21	Canine	Mini Schnauzer	FCE and vasculitis
05.08.21	N273/21	Canine	Rough Collie	Choking
05.08.21	N273/21	Canine	Collie	Choking (asphyxia)
06.08.21	N274/21	Equine	Sport Horse	Peritonitis and duodenal rupture
06.08.21	N275/21	Canine	Labrador	Endocarditis, icterus, renal infarcts
06.08.21	N276/21	Ovine	NA	Emaciation, anaemia and parasites
09.08.21	N277/21	Canine	Husky	Renal and cardiac infarcts
09.08.21	N280/21	Canine	French Bulldog	Pneumonia
10.08.21	N282/21	WILD	Grey seal	Meningoencephalitis
11.08.21	N283/21	Bovine	Jersey	Ruminal impaction and ileus
12.08.21	N285/21	Caprine	NA	Bronchopneumonia (*Pasteurella multocida or Mannheimia haemolytica*) - presumed CAEV
13.08.21	N286/21	Zoo	Ibis	NSF
30.08.21	N308/21	Canine	Border Terrier	Hepatitis
30.08.21	N310/21	Canine	Golden Retriever	GDV
31.08.21	N311/21	Caprine	NA	Fracture

31.08.21	N312/21	Caprine	Texel	Effusion, oedema and systemic vasculitis
02.09.21	N313/21	Canine	Cross	Haemorrhagic enteritis
02.09.21	N314/21	Lagomorph	NA	RHD
01.09.21	N318/21	Canine	Wolfhound	Rickets vs. dwarfism
02.09.21	N319/21	Lagomorph	NA	RHD
03.09.21	N321/21	Feline	Ragdoll	Cat flu (feline caliciviral infection with a possible secondary bacterial infection)
21.09.21	N351/21	WILD	Harbour seal	Trauma
21.09.21	N356/21	Feline	DLH	Pyothrax
21.09.21	N354/21	Bovine	NA	Abomasal ulcers
28.09.21	N368/21	WILD	Harbour seal	NSF
28.09.21	N369/21	Avian	Chinese Pheasant	NSF
29.09.21	N370/21	Zoo	Meerkat	Toxoplasmosis
18.10.21	N397/21	Feline	DSH	Icterus and chronic kidney infarcts
18.10.21	N399/21	Canine	Cross	Onion intoxication
19.10.21	N401/21	Avian	Pigeon	NSF
20.10.21	N403/21	Canine	Chihuahua	Degenerative changes - immune-mediated diseases, or degenerative changes in the vertebral column such as intervertebral disc disease
21.10.21	N404/21	Canine	Foxhound	NSF
27.10.21	N406/21	Canine	Pomeranian	Internal hydrocephalus
27.10.21	N409/21	Canine	Cocker Spaniel	Leptospirosis
27.10.21	N412/21	Avian	Brahman Chicken	NSF
28.10.21	N413/21	Bovine	Friesian	Urogenital malformation
28.10.21	N414/21	Canine	Boxer	Endocarditis
07.03.22	N106/22	Feline	DSH	Endocardial and pulmonary Fibrosis
07.03.22	N107/22	Canine	Bulldog	Pneumonia and pituitary tumour
07.03.22	N110/22	Canine	Spaniel	Marked necrohaemorrhagic enteritis
09.03.22	N116/22	ZOO	Phyton	Significant inflammatory lesion within the oviduct (likely egg-binding)

08.03.22	N117/22	Canine	French Bulldog	Systemic inflammatory process (septicaemia)
08.03.22	N118/22	Canine	French Bulldog	Cerebral perivasculitis and pneumonia
09.03.22	N120/22	Porcine	Pig	Gastric ulceration
09.03.22	N122/22	ZOO	Tiger	DJD, CKD
14.03.22	N123/22	Bovine	Friesian	Severe bacterial pneumonia and systemic inflammatory process
14.03.22	N125/22	Bovine	Belgian Blue	Peritonitis and abomasal ulcer
14.03.22	N127/22	Ovine	NA	Moderate myocarditis, moderate to severe pulmonary oedema and hepatocyte atrophy
15.03.22	N128/22	Bovine	Limousin	Adrenal cortical atrophy and prolonged gestation
16.03.22	N129/22	Ovine	Texel	Severe hypoxia and low PCV (hypovolemia)
16.03.22	N132/22	Feline	DSH	Cardiomyopathy
16.03.22	N134/22	Canine	Boxer	Enteritis (presumed Parvo) and DIC
05.04.22	N164/22	Canine	Vizsla	Acute haemorrhagic gastroenteritis
05.04.22	N168/22	Canine	Whippet	Cardiomyopathy
06.04.22	N169/22	Ovine	NA	Necrotising and haemorrhagic enterocolitis and toxaemia
06.04.22	N170/22	Equine	Shetland Pony	Penetrating wound and infection
07.04.22	N171/22	Canine	JRT	Adenocarcinoma likely of hepatic origin (cholangiocellular)
08.04.22	N172/22	Bovine	Friesian	Severe enteritis associated with *Sarcina* spp., fungi and Giardia
08.04.22	N173/22	Canine	Rottweiler	Hemangiosarcoma
11.04.22	N175/22	Canine	French Bulldog	NSF
11.04.22	N176/22	ZOO	Tricolour Squirrel	Yersiniosis
12.04.22	N178/22	Porcine	Potbellied Pig	Soft tissue sarcoma

13.04.22	N180/22	Feline	DSH	Lymphoma
20.04.22	N184/22	Avian	Amazon	Fatty liver disease and atherosclerosis
21.04.22	N185/22	Canine	Lurcher	Ethylene glycol poisoning and acute tubular injury
22.04.22	N187/22	Equine	Irish Sport Horse	Valvular endocarditis with consequently embolic nephritis and pulmonary infarct
22.04.22	N188/22	Avian	Chicken	Osteoporosis
25.04.22	N190/22	Equine	Donkey	NSF
31.05.22	N258/22	Bovine	Parathenaise	Atrial septum defect and cardiomegaly
31.05.22	N262/22	Bovine	Limousin	Bronchopneumonia
01.06.22	N263/22	Camelidae	Cria	Meningitis
01.06.22	N264/22	Caprine	NA	Myocarditis
02.06.22	N265/22	WILD	Grey seal	Skin disease
03.06.22	N276/22	Canine	Cavachon	*Angiostrongylus* - cerebral bleeding
03.06.22	N277/22	Equine	Donkey	Old age and liver failure
07.06.22	N282/22	Canine	Rottweiler	Perforating stomach ulcer
09.06.22	N283/22	WILD	Grey seal	Kidney stones
08.06.22	N284/22	WILD	Grey seal	Pulmonary emphysema
08.06.22	N285/22	WILD	Harbour seal	Malnutrition
08.06.22	N286/22	WILD	Harbour seal	Malnutrition
08.06.22	N287/22	WILD	Harbour seal	Suppurative myositis
09.06.22	N290/22	WILD	Grey seal	Trauma
09.06.22	N291/22	WILD	Grey seal	Marked ulcerative stomatitis
09.06.22	N292/22	WILD	Grey seal	Severe ulcerative stomatitis
10.06.22	N293/22	WILD	Grey seal	Colitis
10.06.22	N294/22	WILD	Grey seal	Bacterial nephritis, glossitis - possibly viral, ophthalmic dysplasia
10.06.22	N295/22	WILD	Grey seal	Septicaemia
10.06.22	N296/22	WILD	Grey seal	Trauma and systemic infection
14.06.22	N305/22	WILD	Grey seal	Pneumonia and endocarditis
14.06.22	N306/22	WILD	Grey seal	Suppurative meningoencephalitis
13.06.22	N307/22	WILD	Harbour seal	Jaundice (suspected leptospirosis case)
14.06.22	N308/22	WILD	Grey seal	Emaciation
13.06.22	N309/22	WILD	Grey seal	Ulcerative stomatitis
13.06.22	N310/22	WILD	Grey seal	Bronchopneumonia

13.06.22	N311/22	WILD	Grey seal	Trauma
14.06.22	N312/22	WILD	Grey seal	Trauma
16.06.22	N314/22	WILD	Grey seal	Peritonitis
17.06.22	N315/22	WILD	Grey seal	Abscess
16.06.22	N316/22	WILD	Harbour seal	Emaciation, bacterial dermatitis
15.06.22	N318/22	WILD	Grey seal	Emaciation
15.06.22	N319/22	WILD	Grey seal	Bacterial severe pneumonia and nephritis
17.06.22	N321/22	WILD	Grey seal	Trauma, emaciation
16.06.22	N337/22	Canine	Bolognese	Cardiomyopathy with systemic oedema and congestion
05.07.22	N353/22	Lagomorph	NA	Severe cardiac fibrosis
05.07.22	N354/22	Feline	DSH	Trauma
08.08.22	N387/22	Canine	Bichon Frise	Extrahepatic shunt and hepatic encephalopathy
08.08.22	N389/22	Camelidae	Alpaca	Endocarditis, septicaemia, affecting both liver and kidney
09.08.22	N390/22	Feline	DSH	Trauma
10.08.22	N391/22	Lagomorph	NA	Presumed heart failure
20.09.22	N453/22	Feline	DSH	Feline parvovirus infection (panleukopenia) and 2nd bacteriaemia
21.09.22	N456/22	ZOO	Binturong	Longstanding dissecting myocardial fibrosis
28.09.22	N462/22	Canine	Golden Retriever	Parvovirus infection
29.09.22	N463/22	Lagomorph	NA	RHDV type 2, several significant pathogens (*Bordetella* sp., *Klebsiella* sp. and *Staphylococcus* sp.), *Encephalitozoon cuniculi, Eimeria stiedae*
10.10.22	N478/22	Canine	Cross	Myocardial infarct; bacterial infection; heart failure
10.10.22	N479/22	Canine	Cocker Spaniel	Angiostrongyliasis
10.10.22	N480/22	Bovine	Friesian	Johne's disease
11.10.22	N481/22	Feline	Ragdoll	Feline panleukopenia virus and bacterial bronchopneumonia

12.10.22	N482/22	Ovine	NA	Viral pneumonia
13.10.22	N483/22	Laboratory	Mouse	Lymphoma
13.10.22	N484/22	Ovine	NA	Endoparasitism.
14.11.22	N532/22	Porcine	Potbellied Pig	Enteritis and hernia with infarction, multifocal thrombi (likely DIC), necrotising, eosinophilic enteritis, mild interstitial pneumonia
15.11.22	N535/22	Bovine	Jersey	Bronchopneumonia with involvement of both lungworm (patent stage) and *Mannheimia haemolytica.*
17.11.22	N538/22	Canine	Beagle	Urothelial (aka transitional) cell carcinoma
17.11.22	N540/22	Avian	Finch	Septicaemia
28.11.22	N558/22	Canine	Labrador	Marked dorsal hematoma, interstitial pneumonia, unknown connection
28.11.22	N560/22	WILD	Grey seal	Bite wounds and peritonitis
29.11.22	N563/22	Canine	Westi X	Neoplasia (neuroendocrine pattern/appearance)
29.11.22	N564/22	Equine	Warmblood	Colon necrosis with rupture
30.11.22	N565/22	Canine	Pomeranian	Pneumonia
30.11.22	N566/22	Canine	Boxer	Choroid plexus papilloma.
30.11.22	N568/22	Canine	Dogue de Bordeaux	Multiple endocrine neoplasia
01.12.22	N569/22	Ovine	NA	Acute infection with larvae of *Fasciola*
01.12.22	N571/22	Equine	Donkey	Enteritis
02.12.22	N572/22	WILD	Grey seal	Omphalophlebitis
02.12.22	N573/22	Equine	Sport Horse	Face tumour
28.02.23	N100/23	WILD	Grey seal	Omphaloplebitis
	N101/23	WILD	Grey seal	The cause of death could not be determined
01.03.23	N102/23	WILD	Grey seal	Aspiration pneumonia, megaoesophagus
02.03.23	N103/23	WILD	Grey seal	Trauma, fractur mandibula
28.02.23	N104/23	WILD	Grey seal	Bite wounds
02.03.23	N105/23	WILD	Harbour seal	Heart worms

	N106/23	WILD	Harbour seal	Emaciation, megaoesophagus
01.03.23	N107/23	WILD	Grey seal	Emaciation
07.03.23	N128/23	Canine	French Bulldog	Aspiration pneumonia
08.03.23	N133/23	Bovine	NA	Marked, necrotising, bacterial placentitis and atresia coli
	N119/23	WILD	Grey seal	Ulcerative stomatitis
	N120/23	WILD	Grey seal	Presumed septicaemia
	N121/23	WILD	Grey seal	Poor body condition
	N122/23	WILD	Grey seal	Suppurative bronchopneumonia
10.03.23	N129/23	WILD	Grey seal	Osteolysis and abscess
09.03.23	N136/23	Zoo	Ring-tailed Mungicoati	Cardiomyopathy, bilateral DJD of stifles, lumbar spondylosis, deposition of amyloid in thyroid gland
09.03.23	N130/23	WILD	Grey seal	Pyothorax
10.03.23	N139/23	Equine	Donkey	Sarcoids associated with penile sheath, chronic kidney infarction
23.03.23	N153/23	WILD	Grey seal	Hepatic necrosis
24.03.23	N160/23	WILD	Grey seal	Pneumonia
23.03.23	N161/23	WILD	Grey seal	Poor body condition
24.03.23	N162/23	WILD	Harbour seal	Presumed septicaemia, pneumonia with interstitial emphysema
27.03.23	N170/23	Canine	Stafford Bull Terrier X	Likely urothelial cell carcinoma
28.03.23	N175/23	Feline	DSH	
30.03.23	N177/23	WILD	Grey seal	Deep wound with open joint capsule
31.03.23	N178/23	WILD	Grey seal	Pneumonia and parasites
31.03.23	N179/23	WILD	Grey seal	Hepatitis, gastritis, peritonitis, enteritis and parasites
29.03.23	N180/23	Canine	Yorkie	Extrahepatic shunt
30.03.23	N183/23	Amphibia	Frog	NSF
14.04.23	N209/23	WILD	Harbour seal	Pneumonia and gastric obstraction
17.04.23	N210/23	WILD	Harbour seal	Hepatitis, gastritis, pneumonia and parasites
17.04.23	N212/23	Equine	Thoroughbred	Ruptured tendon

18.04.23	N211/23	WILD	Grey seal	Emaciation
17.04.23	N213/23	Equine	Sport Horse	Omphaloplebitis and osteomyelitis
17.04.23	N214/23	Bovine	Frisian	Johne's disease
18.04.23	N215/23	Feline	DSH	Trauma
18.04.23	N216/23	Canine	Maltese X Shih Tzu	Pituitary adenoma
20.04.23	N217/23	WILD	Grey seal	Poor nutrition state and presumed gastric impaction
19.04.23	N218/23	Bovine	Frisian	Bronchopneumonia
19.04.23	N219/23	Avian	Lovebird	NSF
20.04.23	N221/23	Canine	Cross	CNS tumour
20.04.23	N222/23	Avian	Hen	Systemic disease of unknown ethology
24.04.23	N223/23	WILD	Grey seal	Hind limb malformation
24.04.23	N224/23	WILD	Grey seal	Possibly septicaemia, gastric impaction and megaoesophagus
28.04.23	N238/23	WILD	Grey seal	Pneumonia and parasites
28.04.23	N239/23	WILD	Grey seal	Gastric perforation by foreign body
04.05.23	N245/23	WILD	Harbour seal	Possibly septicaemia
03.05.23	N246/23	WILD	Grey seal	Emaciation
02.05.23	N247/23	WILD	Grey seal	Pneumonia and parasites
02.05.23	N249/23	Equine	Trotter	Bacterial typhlocolitis and hepatitis
03.05.23	N251/23	Feline	DSH	Ethylene glycol poisoning
03.05.23	N252/23	Feline	DSH	Ethylene glycol poisoning
04.05.23	N255/23	Canine	Cross	Cause of death could not be determined
04.05.23	N256/23	Equine	Sport Horse	Severe synovitis and arthritis
05.05.23	N257/23	WILD	Common Dolphin	Pox lesions and malnutrition
16.05.23	N275/23	WILD	Grey seal	Pneumonia and parasites
16.05.23	N276/23	WILD	Grey seal	Presumed septicaemia
30.05.23	N299/23	Zoo	Penguin	Septicaemia
31.05.23	N301/23	Canine	Whippet	Cardiovascular component or/and an immune mediated process
31.05.23	N302/23	Feline	Asian Shorthair	Acute hepatic necrosis with a toxic/hypoxic pattern Feline infectious peritonitis

02.06.23	N303/23	Equine	Sport Horse	Umbilical hernia
02.06.23	N304/23	Equine	Thoroughbred	Myositis with systemic spread
19.06.23	N328/23	Zoo	Capuchin	Viral or immune mediated systemic inflammation
19.06.23	N329/23	Zoo	Meerkat	Fungal pneumonia
22.06.23	N333/23	Canine	Cross	Bacterial infect
22.06.23	N334/23	Equine	Thoroughbred	NSF
23.06.23	N335/23	Avian	Capercaillie	*Salmonella* septicaemia
23.06.23	N336/23	Canine	Great Dane	Severe pneumonia and pyothorax with *E. coli* and *Mycoplasma* sp.

Biopsies

Date	Biopsy Number	Species family	Breed	Diagnosis
01.09.20	182/20	Canine	Rhodesian Ridgeback	Spleen: megakaryoblastic leukaemia Testicle: Both seminoma and interstitial (Leydig cell) tumour present in left testicle
01.09.20	20pa72	Canine	NA	Anaplastic mammary carcinoma with vasculature invasion
01.09.20	588/20	Canine	Springer Spaniel	Benign mixed mammary tumour
01.09.20	20pa106	Canine	Cocker Spaniel	Cutaneous mast cell tumour, low-grade (Kiupel)/grade 2 (Patniak)
01.09.20	20PA108	Canine	NA	Trichoblastoma, ribbon/medusoid type (benign follicular tumour)
01.09.20	911/20	Canine	Cavalier King Charles	Multifocal to coalescing, marked suppurative and proliferative stomatitis/gingivitis
01.09.20	913/20	Canine		Perianal (hepatoid) gland adenoma
01.09.20	916/20	Canine	GSD	Marked lympho-histiocytic conjunctivitis
01.09.20	983/20	Canine	JRT	Perianal (hepatoid) gland adenoma
01.09.20	984/20	Feline	Exotic Shorthair	Feline apocrine ductular adenoma
01.09.20	987/20	Canine	Bichon	Nodular sebaceous hyperplasia
01.09.20	1010/20	Canine	Springer Spaniel	Low grade mast cell tumours
01.09.20	1011/20	Canine	Retriever	Osteosarcoma
01.09.20	1012/20	Canine	Cavalier King X	Chronic fibrosing granulomatous panniculitis with central fat necrosis
01.09.20	1094/20	Canine	Yorkshire Terrier	Grade I (on a I-III scale) soft tissue sarcoma (possibly histiocytic? or fibroblastic? DDX: amelanotic melanoma
01.09.20	1095/20	Canine	Labrador X	Fibroma/myxoma
26.01.21	45/21	Feline	DSH	Fibrosarcoma
26.01.21	69/21	Canine	French Bulldog	Cutaneous histiocytoma

26.01.21	72/21	Canine	Labrador	Dermal melanocytoma
26.01.21	74/21	Canine	Cavachon	Mammary carcinoma (tubular to solid sub-type, Grade II on a I-III scale)
26.01.21	76/21	Canine	Yorkshire Terrier	Mammary fibroadenoma
26.01.21	77/21	Canine	Cavalier King Charles	Lymphoma/lymphosarcoma: diffuse, large cell, indolent grade sub-type
05.02.21	134/21	Canine	Boston Terrier	Subcutaneous mast cell tumour
05.02.21	135/21	Canine	Labrador	Acrochordonous plaque with marked hyperkeratosis and comedones and superficial yeasts
05.02.21	136/21	Canine	Springer Spaniel	Lipoma
05.02.21	137/21	Canine	Labrador	Mild chronic lymphocytic gastritis with mild fibrosis and oedema; mild to moderate lymphoplasmacytic enteritis
22.02.21	179/21	Equine	Sport Horse	Mild to moderate chronic lympho-plasmocytic proctitis with fibrosis
22.02.21	183/21	Canine	Yorkshire Terrier	Interstitial (Leydig) cell tumour
22.02.21	184/21	Canine	Spaniel	Sebaceous epithelioma
22.02.21	187/21	Canine	Collie	Collagenous hamartoma
22.02.21	188/21	Canine	JRT	Marked pyogranulomatous conjunctivitis with necrosis and focal vasculitis
22.02.21	191/21	Equine	Sport Horse	Squamous cell carcinoma
22.03.21	292/21	Canine	Boxer	Malignant poorly differentiated neoplasm, exact type uncertain and Spindle-cell neoplasm
22.03.21	303/21	Canine	Siberian Husky	Perianal (hepatoid) gland adenoma
22.03.21	305/21	Canine	Boxer	Haemangioma

22.03.21	306/21	Canine	Stafford	Mild lymphoplasmacytic dermatitis with surface epithelial thinning and scaling, sebaceous atrophy and rare intrafollicular demodex mites
22.03.21	307/21	Canine	Boxer	Cutaneous mast cell tumour, low grade
22.03.21	308/21	Canine	Cocker Spaniel	Mammary carcinoma, grade 1 (low-grade), complex and mixed with cartilaginous differentiation.
08.04.21	367/21	Canine	JRT	Deep Pyoderma (marked, neutrophilic, plasmacytic and histiocytic dermatitis with furunculosis and intra corneal neutrophilic pustules)
08.04.21	371/21	Canine	Husky-Collie-X	Adenomatous polyps with carcinoma in situ and marked, diffuse plasmocytic to neutrophilic proctitis and moderate plasmacytic colitis
08.04.21	378/21	Canine	Cocker Spaniel	Moderate lymphoplasmatytic colitis Lymphoma, low-grade, small cell type.
08.04.21	390/21	Feline	DSH	Moderate suppurative rhinitis
08.04.21	21pa53	Canine	NA	Lipoma, mild suppurative cellulitis
08.04.21	21pa54	Canine	NA	Subcutaneous mast cell tumour
	21pa130	Canine	NA	NSF
08.04.21	21pa131	Canine	NA	Multifocal marked lymphoplasmacytic, suppurative meningoencephalitis with vasculitis and haemorrhages and intranuclear inclusion bodies in endothelial cells and macrophages
08.04.21	21pm303	Canine	NA	Marked hepatic necrosis and haemorrhages with moderate neutrophilic hepatitis and suspect protozoal bradyzoites / tachyzoites and multifocal mild cortical haemorrhages and tubular necrosis
20.04.21	21pm287	Canine	NA	Acute interstitial pneumonia - aetiology unclear; potential causes would include

				protozoa (neospora ?) or bacterial septicaemia
20.04.21	427/21	Canine	Jug	Lymphoplasmacytic gastroenteritis
20.04.21	428/21	Canine	Golden Retriever	Low grade mast cell tumour
20.04.21	429/21	Canine	Labrador	Benign collagenous tumour (i.e. fibroma or collagenous hamartoma)
20.04.21	430/21	Canine	Pug	Subcutaneous mast cell tumour
20.04.21	431/21	Canine	Collie	Active cholangio-hepatitis
01.06.21	620/21	Canine	Stafford	Subcutaneous mast cell tumour
01.06.21	621/21	Canine	Labrador	Bacterial-mediated necrotising splenitis; ongoing bacterial embolism (septicaemia); impedance to bile flow and overall point to a possible toxic/metabolic aetiology
01.06.21	628/21	Canine	Rottweiler	Severe and deep-seated pyogranulomatous inflammation with likely bacterial involvement
01.06.21	630/21	Canine	West Highland Terrier	Adenocarcinoma that appears to originate from the basal layer of the oral epithelium
01.06.21	637/21	Canine	Bichon	Simple mammary adenoma
01.06.21	639/21	Canine	GSD	Complex mammary carcinoma
15.06.21	671/21	Canine	Springer Spaniel	Grade I (low grade) soft tissue sarcoma
15.06.21	674/21	Canine	Collie	Grade I (low grade) soft tissue sarcoma Collagen nevus
15.06.21	680/21	Canine	Scottish Terrier	Dermal atrophy, calcification of basement membranes, epidermal hyperplasia, cellular crust and perivascular dermatitis
15.06.21	681/21	Canine	Irish Red Setter	Mammary gland: simple mammary adenoma
06.07.21	764/21	Bovine	NA	Severe granulomatous dermatitis with acid-fast bacteria associated with lesions
06.07.21	766/21	Canine	Rottweiler	Grade I soft tissue sarcoma (likely fibrosarcoma)

06.07.21	768/21	Canine	Shih Tzu	Apocrine ductular carcinoma
06.07.21	770/21	Canine	JRT	Ceruminous adenoma
06.07.21	772/21	Canine	Labrador	Fibromatous epulis
06.07.21	777/21	Canine	Chow Chow	Lymphoplasmacytic colitis
22.09.21	1150/21	Canine	American Bulldog	Mild mucosal hyperplasia and submucosal oedema
22.09.21	1152/21	Canine	Labrador	Apocrine cystadenoma
22.09.21	1154/21	Feline	DSH	Eosinophilic granulomatous and proliferative glossitis, with marked ulceration and bacteria on the surface
22.09.21	1156/21	Canine	GSDxCollie	Visceral haemangiosarcoma
22.09.21	1157/21	Canine	Collie	Cutaneous lipoma
05.10.21	1200/21	Canine	Springer Spaniel	Grade I soft tissue sarcoma
05.10.21	1202/21	Canine	Kerry Blue Terrier	Matrical cyst & Squamous Cell Carcinoma
05.10.21	1209/21	Canine	Collie X	Meibomian gland hyperplasia and rupture with release of glandular content and inflammation
05.10.21	1210/21	Canine	JRT	Fibroadnexal dysplasia secondary to bacterial follicultits/furunculosis
05.10.21	1213/21	Feline	DSH	Intermediate grade soft tissue sarcoma
05.10.21	1215/21	Canine	Pug x Cavalier King Charles	Urothelial (transitional cell) carcinoma
12.10.21	1233/21	Feline	NA	Eosinophilic granulomatous and proliferative glossitis with ulceration
12.10.21	1234/21	Canine	Cockapoo	Necrotising and lymphohistiocytic steatitis with haemorrhage and granulation tissue formation
12.10.21	1235/21	Canine	Bichon	Cutaneous histiocytoma
12.10.21	1236/21	Canine	Boxer	Cutaneous histiocytoma

01.03.22	221/22	Canine	Border Collie	NSF
01.03.22	226/22	Canine	Cockapoo	Mammary gland: intraductal papillary adenoma
01.03.22	227/22	Canine	JRT	Grade I (on a scale from I to III of increasing malignancy) soft tissue sarcoma - likely perivascular wall tumour
01.03.22	228/22	Canine	Shih Tzu	Low grade soft tissue sarcoma (likely histiocytic)
01.03.22	229/22	Canine	Japanese Spitz	Mild lymphoplasmacytic gastroenteritis
01.03.22	230/22	Canine	Cocker Spaniel	Mild-moderate lymphoplasmacytic enteritis
01.03.22	235/22	Equine	Cob	Preputial papilloma
03.03.22	238/22	Canine	Spaniel Cross	Low grade (grade I) soft tissue sarcoma
03.03.22	240/22	Canine	Wheaten Terrier	Mild-moderate lymphoplasmacytic enterocolitis
03.03.22	242/22	Canine	Labrador	Mammary chondrosarcoma
22.03.22	286/22	WILD	Grey Seal	Proliferative and neutrophilic dermatitis with intralesional fungi and bacteria
22.03.22	294/22	Feline	Ragdoll	NSG
22.03.22	303/22	Canine	Golden retriever	Cutaneous histiocytoma
22.03.22	304/22	Canine	Yorkshire Terrier	Cutaneous histiocytoma
22.03.22	305/22	Feline	DSH	Diffuse iris melanoma
22.03.22	306/22	Canine	Retriever	Cutaneous histiocytoma
22.03.22	307/22	Canine	PugxBeagle	Low-grade mast cell tumour
22.03.22	309/22	Canine	Yorkshire Terrier	Cystic endometrial hyperplasia
22.03.22	311/22	Canine	Scottish Terrier	Pyogranulomatous panniculitis
22.03.22	312/22	Canine	Bichon frise	Trichoblastoma and perianal (hepatoid) gland adenoma

07.06.22	608/22	Canine	Collie	Carcinoma, likely a metastasis from urothelial cell carcinoma
07.06.22	612/22	Canine	Terrier	Moderately severe lymphoplasmacytic enterocolitis
07.06.22	616/22	Canine	Yorkshire Terrier	NSF
07.06.22	619/22	Feline	DSH	Feline eosinophilic plaque/granulomatous pododermatitis
24.08.22	891/22	Canine	Labrador	Subcutaneous haemangioma
24.08.22	892/22	Canine	JRT	Mast cell tumour, high grade
24.08.22	893/22	Canine	Collie	Moderate, lymphoplasmacytic and neutrophilic panniculitis with haemorrhage and granulation tissue.
24.08.22	896/22	Canine	Doberman	Moderate, plasmacytic dermatitis and perihidradenitis with epidermal hyperplasia, hyperkeratosis and dermal fibrosis (interdigital dermatitis/lick dermatitis)
24.08.22	898/22	Canine	Cross	Regressing cutaneous histiocytoma
24.08.22	899/22	Canine	Boxer	Mast cell tumour, high grade
04.10.22	1043/22	Canine	Labrador	Interstitial cell tumour and Sertoli cell tumour
04.10.22	1047/22	Canine	Setter Springer X	Canine clitoral (adeno)carcinoma; intermediate grade soft tissue sarcoma; chronic, pyogranulomatous, marked dermatitis
04.10.22	1050//2	Canine	Terrier	Trichoepithelioma, cystic and pyogranulomatous dermatitis
04.10.22	1053/22	Canine	GSD	Suspect haemangiosarcoma, haematoma, thrombosis, necrosis and extramedullary haematopoiesis
04.10.22	1058/22	Canine	Terrier	Low grade soft tissue sarcoma
04.10.22	1059/22	Canine	French Bulldog	Mild, multifocal, subacute, folliculitis and dermatitis with numerous intralesional fungal arthrospores

26.10.22	1135/22	Canine	Boxer	Islet cell carcinoma (malignant insulinoma)
26.10.22	1140/22	Equine	Pony	Very mild lymphoplasmacytic enteritis
26.10.22	1141/22	Canine	Border Collie	Fibroadnexal dysplasia
26.10.22	1142/22	Canine	Bernese x Pyrenean	Osteosarcoma
26.10.22	1145/22	Canine	Boxer	Mild eosinophilic enteritis
21.03.23	285/23	Canine	Lab x GSD	Cutaneous histiocytoma
21.03.23	286/23	Feline	DSH	Fibrosarcoma (intermediate grade).
21.03.23	287/23	Canine	Terrier	Follicular (matrical) cyst
21.03.23	290/23	Canine	Cockapoo	Lipoma
21.03.23	293/23	Canine	Springer Spaniel	Pancreatic ductular carcinoma and neuroendocrine tumor
12.04.23	380/23	Canine	Akita	Chondroblastic osteosarcoma
12.04.23	381/23	Canine	Golden Retriever	Mammary carcinoma, mixed, low-grade with widespread osseous metaplasia
12.04.23	384/23	Feline	DSH	Tubular carcinoma, grade-2, with lymphatic invasion
12.04.23	386/23	Canine	JRT	Marked neutrophilic lymphoplasmacytic rhinitis with squamous metaplasia
12.04.23	387/23	Canine	Labrador	Anal sac gland carcinoma, tubular type
12.04.23	390/23	Canine	JRT	Soft tissue sarcoma, grade 1 (low-grade)
12.04.23	392/23	Feline	Scottish Fold	Chronic neutrophilic, lymphoplasmacytic and mastocytic rhinitis
16.05.23	534/23	Canine	English Bulldog	Intermediate grade soft tissue sarcoma
16.05.23	536/23	Canine	Dachshound	Chronic active proliferative laryngitis with keratinocyte dysplasia
16.05.23	23PA45	Canine	NA	Intermediate grade soft tissue sarcoma
16.05.23	23PA46	Canine	NA	Amelanotic melanoma
17.05.23	538/23	Feline	DSH	Inflammatory polyp
17.05.23	539/23	Canine	Beagle	Lymphoid hyperplasia
17.05.23	540/23	Feline	DSH	Low grade soft tissue sarcoma
17.05.23	541/23	Canine	Retriever	Early rectal papillary adenocarcinoma
13.06.23	23PA54	Canine	GSD	Benign mixed mammary tumour

13.06.23	23PA55	Canine	Terrier	Vaginal leiomyoma
13.06.23	23PA56	Canine	Malamute	Severe, chronic, ulcerative, pyogranulomatous and lymphoplasmacytic dermatitis with follicular hyperplasia and granulation tissue formation
13.06.23	630/23	Canine	Cocker Spaniel	Moderate necrosuppurative otitis externa
13.06.23	637/23	Canine	Pit Bull X	Extraskeletal osteosarcoma; complex adenoma (adenomyoepithelioma)
13.06.23	638/23	Canine	Cockapoo	(Pyo)granulomatous lymph adenitis
13.06.23	639/23	Canine	Pug	Low grade mast cell tumour
13.06.23	640/23	Canine	Poodle	Complex adenoma (adenomyoepithelioma); lobular hyperplasia with fibrosis
16.06.23	649/23	Canine	GSD	Cutaneous haemangiosarcoma
16.06.23	657/23	Canine	English Setter	Tonsillar squamous cell carcinoma