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Companion Animal Tuberculosis

Clinical Presentations, Outbreak Investigations, Improved Diagnostics & The Early Macrophage Response

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Declaration

I declare that this thesis, entitled *'Companion Animal Tuberculosis: Clinical Presentations, Outbreak Investigations, Improved Diagnostics & The Early Macrophage Response',* has been composed by myself and the work is entirely my own or I have made a substantial contribution to the work, with such contribution being clearly indicated. I declare that the work is orignial research which has not been submitted for any other degree or professional qualification. Where publications are included in this thesis I confirm that, as the author, I retain the copyright to the content of all of the works.

Conor O'Halloran

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Scientific Abstract

Tuberculosis caused by the *Mycobacterium (M.) tuberculosis*-complex (MTBC) of organisms remains one of the most prevalent and deadly infectious diseases of man and other animals. The mycobacteria responsible are a highly conserved group of pleomorphic acid-fast bacilli which cause chronic granulomatous infections. Tuberculous infections in humans and cattle often remain latent for prolonged periods of time before progressing to disease that has severe, negative consequences for the health and welfare of the infected host. Some of the organisms within the MTBC are highly specialised and limited to just a single or small number of host species whereas others, such as *M. bovis,* can infect a broad range of mammals including humans. Companion animals are susceptible to MTBC infections and understanding of the significance and frequency of these infections has grown in recent years. Cats and dogs share unrivalled proximity to their owners and therefore pose a small but real risk for the zoonotic transmission of tuberculous infections. Despite the high frequency of mycobacterial infections observed in companion animals, diagnostic tests to identify the commonly encountered mycobacterial species are lacking.

The first aim of this work was therefore to improve on the currently available diagnostic test methodologies for companion animals. A diagnostic PCR assay was developed and applied to 380 histologically confirmed feline and eight canine mycobacteriosis samples. This novel assay specifically targeted the mycobacterial species most frequently identified by mycobacterial culture (*M. bovis* and *M. microti*) and was optimised for use with formalin-fixed tissue; a prerequisite for the safe handling of tuberculous tissue from companion animals by UK laboratories. The assay was suitable for both feline and canine tissue with a significantly quicker turnaround time, higher rate of test positive results and a significant increase in the proportion of *M. microti* diagnoses compared to culture results.

Since evaluation of cytokines has shown diagnostic potential in other species, this project explored the potential of cytokine profiling in cats for the rapid and sensitive detection of mycobacteriosis. By evaluating serum/plasma from 116 naturally infected cats, this study demonstrated a consistent elevation in the cytokines associated with macrophage activation and antigenic stimulation compared to control cats. Sub-group analysis showed that elevations in PDGF-BB were specifically associated with *M. microti* infections whereas elevated TNF-α sensitively identified cats infected with *M. bovis.*

Investigation of an unprecedented outbreak of *M. bovis* associated with the ingestion of a putatively contaminated raw food product led to the use of the interferon-γ release assay (IGRA) as a screening test for clinically healthy cats, a purpose for which it had not previously been evaluated. Nearly a third of clinically normal IGRA test-positive cats were subsequently found to have structural disease detected by diagnostic imaging. Though this raises questions regarding the specificity of the IGRA in clinically normal cats, it was an invaluable diagnostic tool to evaluate individual cats involved in the outbreak.

The work on feline TB was complemented by similar investigations of canine TB. When an outbreak of *M. bovis* tuberculosis occurred in a kennel of 164 working Foxhounds, a testing strategy to successfully bring the outbreak under control and investigate the cause was developed. Collaborative work undertaken to screen at risk humans exposed to the hounds identified a latently infected person, highlighting the zoonotic risk posed by *M. bovis* infections in companion animals. Eight novel and existing diagnostic testing methodologies were evaluated for use in dogs of which a cell-based IGRA, and three serological tests comprising a novel comparative peptide ELISA, the Chembio DPP VetTB assay and the Idexx *M. bovis* Ab ELISA showed diagnostic potential for canine TB. Additional analysis employing a Bayesian latent class modelling approach revealed that the IGRA developed herein was as sensitive and specific as comparable tests in other species. The serological assays were shown to have markedly lower sensitivity than the IGRA but had higher specificities. All four tests had positive and negative predictive value estimates which indicate that these tests can be informative to clinicians who suspect cases of canine TB.

A review of 1012 cases of canine TB highlighted an apparently lower incidence of infections in dogs compared to cats, but an increased severity of clinical signs when disease occurred. To investigate this discrepancy a protocol to derive macrophages from canine and feline bone marrow was developed. These cells acquired cell surface molecules indicative of a macrophage phenotype during ten days of culture with recombinant CSF-1. The response of primary macrophages and the DH82 canine histiocytic cell line was assessed following stimulation with LPS, infection with *M. bovis* Bacille Calmette Guerin, *M. bovis* AF2122/97 (the reference strain) or a clinical isolate of *M. bovis*. These investigations consistently revealed that DH82 cells do not accurately represent primary canine macrophage biology which was associated with altered morphology, lack of nitrite production and significantly reduced secretion of the pro-inflammatory cytokines IL-6 and TNF-α. Overall, the work presented in this thesis demonstrates novel advancement of the diagnostic methodologies for identifying cases of companion animal mycobacteriosis and in particular cases of TB. It further begins to explore the immunological basis for the clinical differences seen between species that may further contribute to novel testing and treatment strategies in the future.

Lay Summary

Tuberculosis (TB) caused by the *Mycobacterium tuberculosis*-complex (MTBC) of organisms remains one of the most prevalent and deadly infectious diseases of man and other animals. TB infections in humans and cattle often remain latent for prolonged periods of time before progressing to disease. Some of the organisms within the MTBC are highly specialised and limited to just a single or small number of host species whereas others, such as *Mycobacterium bovis,* can infect a broad range of mammals including humans and pets. Our understanding of the significance and frequency of these infections in pet cats, and to some extent in dogs, has grown in recent years. Cats and dogs share unrivalled proximity to their owners and therefore pose a small but real risk for the zoonotic transmission of tuberculous infections. Despite the high frequency of mycobacterial infections observed in companion animals, diagnostic tests to identify the commonly encountered mycobacterial species are lacking.

The aim of this project was to improve on the currently available diagnostic tests for companion animals and in doing so also developed a greater understanding of the immune response of cats and dogs to these infections which will allow future research to explore ways to intervene to treat tuberculosis or even vaccinate cats and dogs against infection.

Abbreviations

1,25-dihydroxyvitamin D_3	$1,25(OH)_2D_3$
10kDa culture filtrate protein	CFP-10
16-23S internal transcribed spacer	ITS
25-hydroxyvitamin D	25(OH)D
65kDa heat shock protein	*hsp65*
6kDa early-secreted antigenic target	ESAT-6
Acid-fast bacilli	AFB
Agriculture and Horticulture Development Board	AHDB
Alkaline phosphatase	ALP
Animal and Plant Health Agency	APHA
Antibody	Ab
Approved finishing unit	AFU
Biologically appropriate raw food	BARF
Body Mass Index	BMI
Bone marrow derived macrophages	BMDM
Bovine serum albumin	BSA
Bovine tuberculosis	bTB
British Cattle Veterinary Association	BCVA
Canine leproid granuloma syndrome	CLG
C-C motif ligand	CCL
Cell mediated immunity	CMI
Central nervous system	CNS
Centre for Disease Control and Prevention	CDC
Cluster of differentiation	CD

Colony forming unit	CFU
Colony stimulating factor	CSF
Computed tomography	CT
Confidence interval	CI
Credibility interval	CrI
Dendritic-cell specific intracellular adhesion molegule non-integrin	DC-SIGN
Department for the Environment, Food and Rural Affairs	DEFRA
Deviance information criterion	DIC
Discrimination between infected and vaccinated animals	DIVA
DNA gyrase	*gyr*
Domestic shorthair [cat]	DSH
Dual path platform	DPP
Enzyme linked immunosorbent assay	ELISA
Every	q
Fas/Fas ligand	FasL
Feline Coronavirus	FCoV
Feline Immunodeficiency virus	FIV
Feline infectious peritonitis	FIP
Feline Leukaemia virus	FeLV
Fine needle aspirate/aspiration	FNA
Fms-like tyrosine kinase 3 ligand	Flt3L
Food standards agency	FSA
Formalin-fixed paraffin-embedded	FFPE
Gastrointestinal	GI
Granulocyte-monocyte colony stimulating factor	GM-CSF
Health protection agency	HPA
Health Protection England	HPE
Health protection team	HPT
High risk area	HRA

Horseradish peroxidase	HRP
Human Immunodeficiency virus	HIV
Inducible nitric oxide synthetase	iNOS
Inflammatory bowel disease	IBD
Insertion sequence	IS
Interferon gamma induced protein	IP-10
Interferon gamma release assay	IGRA
Interferon gamma	IFN-γ
Interleukin	IL
Keratinocyte chemoattractant	KC
Lipopolysaccharide	LPS
Low risk area	LRA
M. bovis bacille Calmette–Guérin	*M. bovis*-BCG
Major histocompatibility complex class II	MHC class II
Masters of Foxhounds Association	MFHA
Median fluorescence intensity	MFI
Member of the Royal College of Veterinary Surgeons	MRCVS
Mitogen-activated protein kinase	MAPK
Monocyte chemoattractant protein	MCP
Monocyte drived macrophages	MDM
Monocyte-macrophage lineage	MML
Mycobacteria other than tuberculosis	MOTT
Mycobacterium	*M.*
Mycobacterium avium subspecies *paratuberculosis*	MAP
Mycobacterium interspersed repetitive unit and variable number of tandem repeats	MIRU-VNTR
Mycobacterium-avium-intracellulare complex	MAC
Mycobacterium-tuberculosis complex	MTBC
National Farmers Union	NFU

Natural killer [cell]	NK
Negative predictive value	NPV
Nitric oxide	NO
Nitric oxide synthase two	NOS2
Non-steroidal anti-inflammatory drugs	NSAID
Nucleotide-binding oligomerisation domain	NOD
Officially tuberculosis free	OTF
Pathogen associated molecular patterns	PAMPS
Pattern recognition receptors	PRR
Per os	PO
Peripheral blood mononuclear cells	PBMC
Personal protective equipment	PPE
Phosphate buffered saline	PBS
Platelet derived growth factor – dimeric for beta units	PDGF-ββ
Polymerase chain reaction	PCR
Positive predictive value	PPV
Post mortem examination	PME
PPD from *M. avium*	PPDA
PPD from *M. bovis*	PPDB
Purified protein derived	PPD
Raw meat based diet	RMBD
Reactive nitrogen species	RNS
Recombinant canine	rc
Recombinant human	rh
Recombinant porcine	rp
Reference interval	RI
Region of [genomic] difference	RD
Registered Veterinary Nurse	RVN
Relative light units	RLU

Restriction fragment length polymorphism	RFLP
Reverse-Transcription quantitative PCR	RT-qPCR
Room temperature	RT
Sensitivity	Se
Single intradermal comparative cervical tuberculin skin test	SICCT
Soluble Fas	sFas
Specificity	Sp
Standard deviation	SD
Stem cell factor	SCF
Stromal derived factor	SDF
Systemic Inflammatory Response Syndrome	SIRS
T cell receptor	TCR
TIR-domain containing adaptor-inducing interferon beta	TRIF
Toll-like receptor	TLR
Transforming growth factor beta	TGF-β
Tuberculin skin test	TST
Tuberculosis	TB
Tumour necrosis factor alpha	TNF-α
Unites States	USA
Visible lesions	VL
Workshop cluster	WC
World Organisation for Animal Health	OIE
Ziehl-Neelsen	ZN
B-subunit of RNA polymerase	*rpob*

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Chapter 1: Introduction to Companion Animal Mycobacterial Disease.

The genus *Mycobacterium* sits within the family Mycobacteriaceae, order Actinomycetales. Mycobacteria are aerobic (or in some cases, microaerophilic), non-spore-forming, non-motile, pleomorphic bacterial rods[1-4]. They are more resistant to heat, extremes of pH, and routine disinfection than almost all other bacteria, properties conferred by their notably thick, hydrophobic cell wall, which contains high concentrations of mycolic acids and lipids[1-4]. The cell wall also provides resistance to decolourisation with acid-alcohol[1-4]. This acid-fast staining property, combined with their morphology, make species of the genus recognisable microscopically, including in histopathological and cytological specimens[1-4].

The genus contains an increasingly recognised number of species that have been documented to cause widely divergent clinical syndromes[1,2]. At one extreme, these species have among them some of the most ancient, notorious, and arguably successful obligate pathogens of both people and animals. The most widely known and best characterised mycobacterial diseases of people are tuberculosis (most commonly caused by *Mycobacterium (M.) tuberculosis*) and leprosy (caused by *M. lepromatosis* and *M. leprae*)[2,5]. Nine thousand-year-old human remains in the Eastern Mediterranean contain evidence of the DNA of mycobacteria belonging to the *M. tuberculosis*-complex (MTBC)[6], and MTBC DNA has been recovered from the diseased bones of a bison trapped in permafrost *circa* 17,000 years ago[7]; Using the most recent methods to exclude environmental contamination has identified MTBC DNA in samples that date from up to *circa* 5000 years ago[8]. The MTBC are of significant global health concern; one third of the current global human population is estimated to be infected with a MTBC organism, of which 5-10% will likely develop active clinical disease[9,10], and it is estimated that up to 85% of all African bovids reside in areas with a high prevalence of poorly controlled bovine tuberculosis[11].

At the other end of the spectrum are numerous potentially pathogenic environmental non-tuberculous mycobacteria (NTM). Being opportunistic pathogens in certain circumstances, they are now recognized as an increasing global public health problem, particularly in immunocompromised patient groups such as cystic fibrosis sufferers[12]. Of these environmental mycobacteria, members of the *M. avium-intracellulare*-complex (MAC) in particular are an emerging threat to human health, with a growing body of evidence that hospital admissions due to NTM-associated pulmonary disease are increasing, particularly in countries with a low incidence of tuberculosis, typically ascribed to increasing numbers of these patients surviving for longer, the use of advanced diagnostic tools, and a larger clinical awareness of the role of NTM in disease[12].

The taxonomy of the genus is notoriously complex, and a major overhaul of current taxonomic nomenclature may soon become necessary. Two primary systems exist for the taxonomic division of the genus. The Runyon scheme was developed in the latter half of the 20th century and has dominated the scientific literature until very recently[13]. It is based on an isolate's laboratory growth characteristics including; substrate usage, growth time until colonies are visible, and subsequent colony phenotype. As it is not possible to grow all species under artificial (*i.e.* laboratory) conditions, and with the advent of advancing molecular techniques (such as next generation whole genome sequencing), there is an increasing departure from the Runyon system towards using molecular phylogeny as the taxonomic basis for speciating the genus[14].

Conceptually, mycobacteria can most easily be divided into two main groups:

1. The members of the MTBC (*M. tuberculosis, M. bovis, M. bovis* bacille Calmette–Guérin [*M. bovis*-BCG] *M. africanum, M. pinnipedii, M. caprae, M. canettii, M. orygis, M. suricattae, M. mungi* and *M. microti*), which are considered obligate intracellular pathogens[15,16].
2. Opportunistic saprophytes which are variably distributed in the environment, especially in soil and water systems (both natural and man-made)[17]. These are generally known as the NTM but are alternatively referred to as 'mycobacteria other

than tuberculosis' (MOTT) or 'atypical' mycobacteria[18]. Of the more than 170 uniquely characterised NTM species, approximately 25 have been shown to be potentially pathogenic in humans and animals[14]. This group can be further subdivided in to (i) slow-growing (e.g. *M. avium*). (ii) rapid-growing (e.g. *M. chelonae*) or (iii) fastidious mycobacteria (e.g. *M. lepraemurium*). The latter group of organisms cannot be cultured by routine laboratory methods and have an enigmatic ecological niche (the so called "fastidious mycobacteria"). Genetic studies of these organisms typically group some of them amongst the slow growers, while others form a separate group containing *M. leprae* (and related bacteria, *M. lepromatosis, Candidatus* 'M. lepraefelis'[19], as well as possibly *M. tarwinense, M. visibile* and the causative agent of bovine mycobacterial thelitis[20]).

Although much is known regarding tuberculous mycobacterial infections in humans and cattle, fewer studies have reported tuberculosis in companion animals. The importance of mycobacterial infections in companion animals has become ever increasingly apparent in the last two decades[19,21-26]. Approximately 1% of all feline biopsy samples submitted for histopathological analysis in the UK have been shown to display changes consistent with mycobacteriosis, and a third of these have demonstrable acid-fast bacilli (AFB) when stained with carbol-fushin using the Ziehl-Neelsen (ZN) method, with a thin rod-like appearance indicative of the presence of mycobacteria[21]. Currently, comparable data do not exist for dogs, but the recognition of canine mycobacteriosis is increasing.

1.1 Feline Mycobacteriosis

1.1.1 Feline Tuberculosis

The MTBC consists of phylogenetically highly related species of mycobacteria capable of causing tuberculosis in man and/or other animals. Of these species, only *M. bovis* and *M. microti* have frequently been detected in cats[22-26]. Successfully cultured samples from cats with suspected mycobacterial infections in the only UK study published to date confirmed that 19% of feline mycobacterial infections were caused by *M. microti* and a further 15% by *M.*

bovis (data reproduced in Table 1.1)[24]. Therefore, any given cat with a mycobacterial infection in the UK has a greater than a third probability of having tuberculosis. Similar data have not been published for other tuberculosis-endemic locations such as the United States (USA).

Table 1.1: Mycobacterial culture results from UK cats. Samples had histological findings indicative of mycobacteriosis and were submitted to the Animal Health and Veterinary Laboratories Agency (now Animal and Plant Health Agency [APHA]) for mycobacterial culture between January 2005 and December 2008[24].

Species of mycobacteria cultured	Number	Percentage of total submissions	Percentage of culture positive submissions
M. microti	63	19	40
M. bovis	52	15	33
M. avium	24	7	15
M. malmoense	4	1	3
M. fortuitum	4	1	3
M. celatum	1	<1	<1
M. intracellulare	1	<1	<1
Unclassified	10	2	6
No growth	180	53	-
Culture positive total	159	47	-
Grand total	339	100	-

M. tuberculosis infection, the leading cause of human tuberculosis, is reported as being 'very rare' in the cat and it is possible that some (if not all) of the small number of cases of feline *M. tuberculosis* infections reported in the literature could, in fact, have been due to other MTBC mycobacteria as historic typing methods are now widely accepted to have been inaccurate for sub-classification of the MTBC[27]. Furthermore, it has been demonstrated that cats have a

natural resistance to disease caused by this pathogen based on experimental challenge studies[28], though the underlying mechanism for this remains unclear.

Consistent strong geographical predispositions exist wthin feline infections with mycobacteria in general. In the UK, *M. bovis* infections are strongly co-incident with where there are high levels of endemic infection in local bovine and wildlife populations such as the south-west of England[24,29]. In comparison, *M. microti* infections are much more common in areas with high prevalence of this infection in the wild rodent population, typically south-east of London, the north of England and throughout Scotland[24,29].

Tuberculosis is most frequently diagnosed in adult male cats with a history of frequent hunting behaviour[24]. The median age at which clinical disease presents is three years for *M. bovis* and eight years for *M. microti.* Unlike human tuberculosis, there is no link between feline MTBC infections and classical immunosuppression caused by exogenous retroviral infections *i.e.* feline immunodeficiency virus (FIV) and feline leukaemia virus (FeLV) infection, with diagnoses of these infections being made as frequently in mycobacteria-infected cats as the general UK cat population.

When the anatomical distribution of lesions is considered in the context that the highest incidence of infections occurs in young hunting cats (particularly those catching small rodents), that data show mice and voles are permissive hosts for *M. microti*[26] and that mice, rats, stoats, mink and ferrets (as well as a wide range of larger mammals) can harbour *M. bovis*[29,30], this cumulatively provides good evidence that cats are infected through injuries sustained while hunting and fighting. Interestingly, however, this does not fully explain the male predilection for clinical disease caused by these infections as female cats are frequently more avid hunters than their male counters which should logically increase their exposure to infection. Whether or not this difference therefore represents sex-linked differences in innate immune function has not been examined to date.

Spoligotyping and/or genotyping of isolates of both *M. microti* and *M. bovis* shows almost perfectly matching geographic distributions of feline, bovine and wildlife strains of these

mycobacteria in the UK[24,29,30]. In the case of *M. bovis,* prey species most probably act as intermediate hosts, picking up infections from environmental sources such as areas around infected badger setts or other contaminated areas where *M. bovis* can persist for extended periods within the environment[30].

Cases of feline tuberculosis appear to have a lower incidence outwith the UK. This may reflect differences in the epidemiology of these organisms – for example, *M. bovis* has been eliminated from much of central Europe, Australia and some states in the USA[31-34]. However, there does appear to be an increase in the incidence of *M. microti* (in companion animals as well as wildlife such as wild boar) in some countries, including France, Switzerland and Germany, where *M. bovis* is no longer considered to be a major concern[31-34].

Cases of tuberculosis have occurred where cats within the same household or in close geographical proximity have presented with clinical signs of active disease within a short time of each other[35,36]. It is difficult to interpret the epidemiological significance of such cases as these cats will share a single source of prey that may harbour infection (e.g. local woodland); it is therefore possible that such infections are independently acquired rather than being representative of cat-to-cat transmission. However, multiple within-household cases have occurred where at least one cat has had no history of outdoor access (Professor Gunn-Moore; unpublished observation). In these cases, cat-to-cat transmission is the putative route of infection, probably by close contact such as grooming. Indeed, direct cat-to-cat transmission was documented in an outbreak of *M. bovis* infection in a group of research cats[37]. It should be noted that the number of these cases remains small and the risk to healthy, immunocompetent cats in contact with an infected cat is generally considered low.

Nosocomial transmission of *M. bovis* has been reported[38]. In one outbreak, the index case, with a purulent ulcerated submandibular lesion, was euthanased and two cats that underwent elective surgery in the same practice soon after developed confirmed tuberculous disease. It is significant to note that the subsequent cases developed severe systemic disease in a much shorter timescale than is typical of disease acquired through sylvatic infection[38]. While only this single well documented instance exists in published literature, this likely reflects under-

recognition in clinical practice. Similarly, although it has not been definitively demonstrated, there is strong circumstantial evidence for humans with active tuberculosis infecting their pet cats[39,40]. This is unsurprising given the small infectious dose of as few as ten mycobacteria such as *M. bovis* required to cause disease in most mammals, including cattle and presumptively also cats[41,42].

Clinical Signs

Most tuberculosis cases present with localised nodular cutaneous disease (Figure 1.1), frequently with a degree of ulceration, and occasionally with a draining sinus tract[23-25]. The lesions are typically distributed around the face, extremities and tail base – the so-called "fight and bite sites" (Figure 1.2). Skin lesions may be accompanied by a localised or occasionally a generalised lymphadenopathy. Lymphadenopathy, usually of the submandibular or popliteal nodes, may alternatively be the only presenting sign (termed an incomplete primary complex)[23].

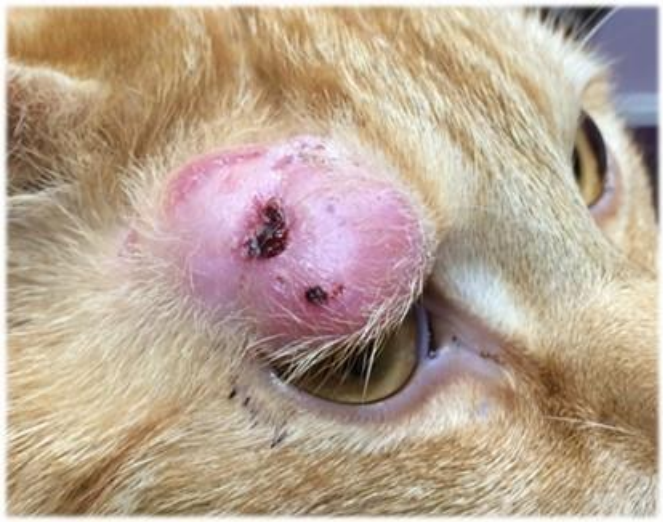

Figure 1.1: A typical alopecic cutaneous nodule (granuloma) above the right eye of a four-year-old, male, neutered, retrovirus negative (*i.e.* negative for FIV and FeLV infections) domestic short hair (DSH) cat caused by *M. bovis* infection. *Image courtesy for Rory Lyndon (Member of the Royal College of Veterinary Surgeons [MRCVS]).*

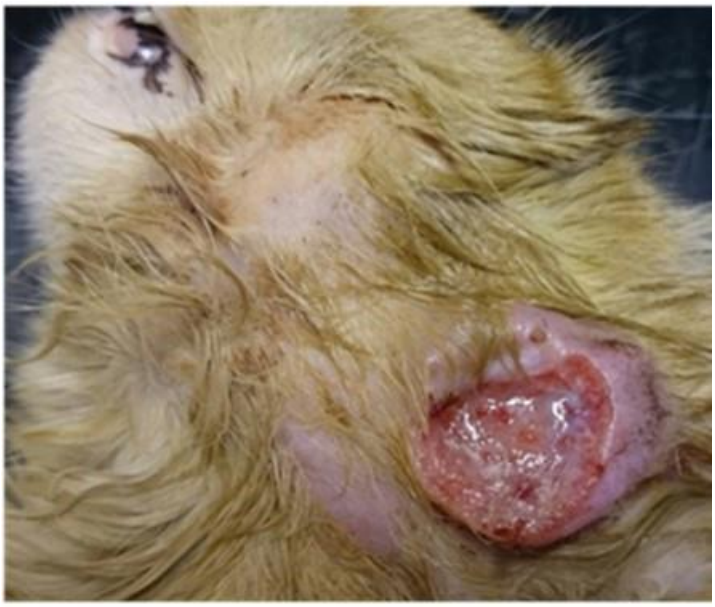

Figure 1.2: A facial granuloma (a “fight and bite site”) over the rostro-lateral aspect of the temporomandibular joint of a four-year-old, female, neutered, retrovirus negative, DSH cat due to *M. microti* infection.

A predominately gastrointestinal (GI) form of the disease exists, where granulomas form in the intestines[24]. There is thickening of the intestine, accompanied by multicentric abdominal lymph node involvement, almost always including the mesenteric nodes. The subsequent intestinal malabsorption causes weight loss, diarrhoea, vomiting and anaemia[24]. This form of the disease was traditionally associated with cats drinking tuberculous cows’ milk and therefore, since the introduction of statutory pasteurisation, this presentation has declined in incidence.

Pulmonary lesions can occur when bacteria are inhaled, resulting in classical tubercle formation in the lungs and associated lymph nodes (e.g. sternal, bronchial and hilar). Much more common, however, is pulmonary disease secondary to the putative haematogenous spread of bacteria from the site of inoculation in the skin. This generates a diffuse interstitial pattern of disease[43] (Figure 1.3) which eventually becomes bronchial and is clinically observable as progressive dyspnoea followed by the development of a soft productive cough. Radiographically this differs from primary pulmonary infection which more frequently causes cavitating lesions[23].

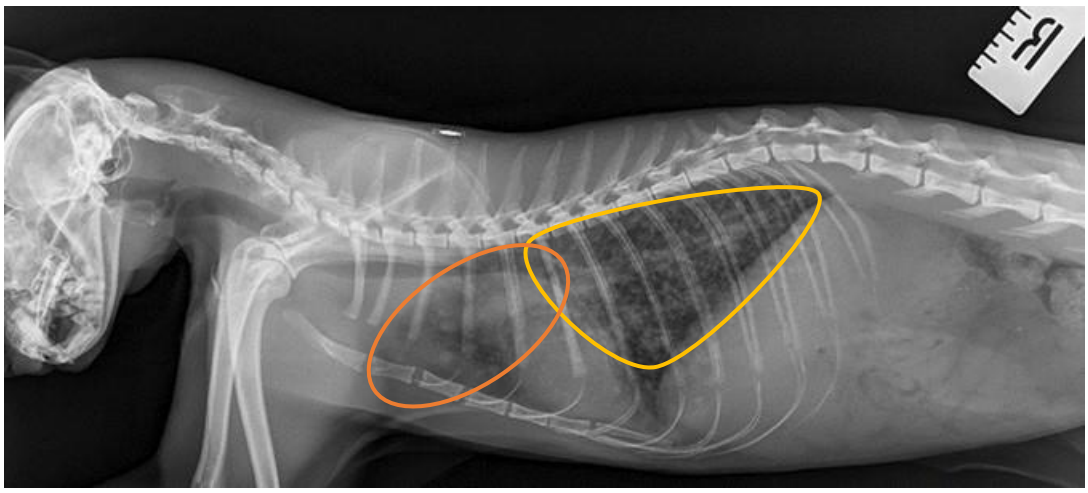

Figure 1.3: A right latero-lateral thoracic radiograph of a five-year-old, male, entire, retrovirus negative, Egyptian Mau cat infected with *M. microti.* The radiograph shows diffuse interstitial pathology with soft tissue opacity (outlined in yellow) plus peribronchial and sternal lymphadenopathy (outlined in orange); these are typical findings associated with secondary pulmonary dissemination of tuberculosis in the cat. *Radiograph courtesy of Emma Campbell (Registered Vterinary Nurse [RVN]).*

Disseminated disease can cause a range of clinical signs, including hepato-splenomegaly, pleural and pericardial effusions, generalised lymphadenopathy, weight loss and pyrexia[24].

Ocular involvement (chorioretinitis[44], conjunctivitis[45]) and tuberculous arthritis/osteomyelitis have been occasionally reported[46,47].

1.1.2 Non-Tuberculous Mycobacteriosis

Slow-growing NTM

These organisms include those whose reference isolates take longer than seven days to culture in laboratory conditions[48]. The MAC organisms are significant within this group as they are the most frequently confirmed NTM infection in companion animals, and are also potential zoonoses[24]. Slow-growing mycobacteria that are known to cause disease in cats include: MAC[49-68] (specifically, *M. avium* subsp. *avium*[69,70], *M. avium* subsp. *hominissuis*[71-73] and *M. intracellulare* [74,75]), *M. genavense*[76], *M. malmoense*[23,77], *M. celatum, M. terrae* complex[78], *M. simiae*[79], *M. xenopi*[80-83], *M. ulcerans*[84], and *M. heckeshornense*[85].

Rapid-growing NTM

These organisms are defined as those whose reference isolates can be grown in laboratory culture conditions within seven days at temperatures of between 24ºC and 45ºC. Documented feline infections include members of the *M. fortuitum* group[23,86-93] (specifically, *M. fortuitum*,[73,94] *M. porcinum*,[95] and *M. alvei*[95]), *M. smegmatis* group[75,87,96,97] (*M. smegmatis sensu stricto*,[73,95] *M. goodii*,[73,88,95] *M. wolinskyi*[73]), *M. chelonae/abscessus* group[86,88,91,98] (including *M. abscessus* subsp. *bolletti* [formerly *M. massiliense*[99]]), *M. mageritense* group,[95] *M. mucogenicum* group,[75] *M. falvenscens,*[86] *M. phlei,*[100] and *M. thermoresistible*.[73,101-103]. Cases have been reported from tropical (Brazil[94]), subtropical (south eastern and south western USA[88,91,100]), and temperate regions, including Australia,[95,97,102] New Zealand,[75] Canada,[75] Finland,[104] Germany,[103] UK[23] and the Netherlands[98,101]. Geographical differences exist with respect to species incidence; most infections in cats in eastern Australia are caused by *M. smegmatis* group (followed by *M. fortuitum*),[87,105-107] whereas, in southwestern USA, cats are most commonly infected with *M. fortuitum* group, followed by *M chelonae/abscessus* group[86,88,90].

Fastidious NTM

To date; *M. lepraemurium* (closely related to *M. avium*)*, Candidatus* “M. tarwinense” (related to the *M. simiae* group), *M. visibile*, and *Candidatus* “M. lepraefelis” (both related to *M. leprae* and *M. lepromatosis*) have been confirmed as capable of causing “feline leprosy syndrome” (FLS) in cats. These infections are reported from New Zealand,[108,109] eastern Australia,[110,111] western Canada,[112,113] the UK,[114,115] south-western USA,[116] the Netherlands[117]. France,[118,119] New Caledonia, Italy,[120] the Greek island of Kythira,[121] and Japan[122].

Similar to the MTBC (as described in Section 1.1.1), the NTM bacteria most often infect cats via presumed cutaneous inoculation, which is reflected in the typical distribution of lesions[23]. Inoculation of the organism directly into subcutaneous adipose tissue appears to increase the severity of rapid-growing NTM disease in cats, to which overweight cats are unsurprisingly predisposed.

Risk factors that have been identified for NTM disease in cats include, the administration of immunosuppressive drugs such as exogenous corticosteroid administration[69], multidrug chemotherapy protocols for lymphoma treatment,[20] cyclosporine treatment in the context of renal transplantation[53], exogenous retroviral infection[76,123] (plus lymphoma in one case[85]), idiopathic αβ-T cell lymphopenia (particularly $CD4^+$ αβ-T-cells)[83], or concurrent comorbidities such as advanced chronic kidney disease and cryptococcosis[50]. Studies of human tuberculosis and NTM infected patients have revealed low body mass index (BMI), specifically a low body fat percentage[124,125], as well decreased interleukin (IL)-10 and interferon gamma (IFN-γ) production capacity by peripheral circulating leukocytes as risk factors for disease following infection[126,127]. Equivalent studies have not been conducted in companion animals, so it is unclear how relevant these factors are to veterinary patients. The MAC infections are seen more frequently in Somali, Abyssinian[51] and Siamese[59] breeds of cat, but there is no recognised breed predisposition for the other NTM.

The MAC organisms have been the most frequently successfully cultured of the NTM from UK cats[23] (Table 1.1); however, the culture data for NTM infections, in general, is inevitably skewed by the fact that fastidious organisms cannot be cultured, and by the routine use of media most sensitive for MTBC culture (e.g. Middlebrook 7H11 OADC) by many reference laboratories in the UK. Therefore, more data need to be collected with regards to the molecular epidemiology of mycobacterial infections in cats on a worldwide basis.

Clinical Signs

Disease caused by NTM is more variable in clinical presentation than tuberculosis, but generally results in (i) (sub)cutaneous nodules, (ii) granulomatous panniculitis, or (iii) disseminated disease. The nodular and disseminated forms can have a very similar presentation and distribution to tuberculous lesions (*i.e.* the head, limbs and trunk, Figure 1.4). Skin nodules can be haired, alopecic or ulcerated but are typically non-painful and freely mobile.

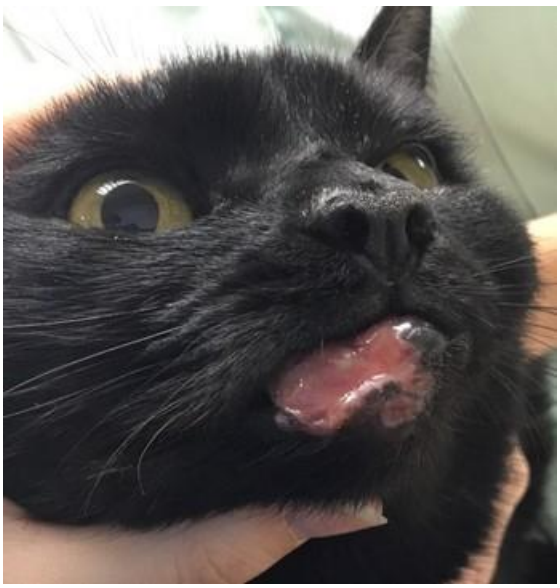

Figure 1.4: An alopecic, erythematous granuloma on the lower lip of a six-year-old, male, neutered, retrovirus negative, DSH cat caused by *M. avium* infection. The lesion is notable for its similarity in gross appearance to tuberculous lesions such as those shown in Figures 1.1 and 1.2. *Image courtesy of Laura de los Santos (RVN).*

Granulomatous panniculitis is the most common manifestation of disease caused by the rapid-growing NTM. [75,86,88-91,96-97,104,107,107-111] It is characterised by multiple punctate drainage tracts and subcutaneous nodules which can coalesce to form large areas of (mostly) non-painful, non-healing ulcerated skin overlaying inflamed fat pads (Figure 1.5). Initially, cats tend to present with a circumscribed plaque or nodule of the skin and subcutis.

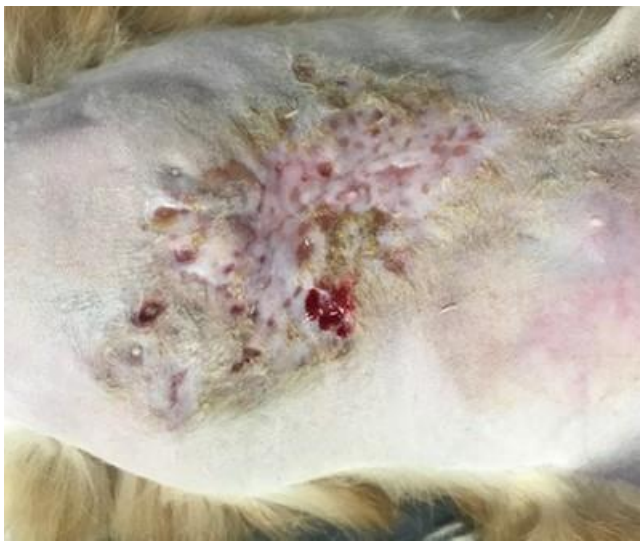

Figure 1.5: Extensive subcutaneous panniculitis affecting the (shaved) ventral skin of an eight-year-old, male, neutered, retrovirus negative, DSH cat caused by *M. smegmatis* infection. *Image courtesy of Faye Swinborne (MRCVS, Willows Referral Centre).*

Later in the clinical course, the subcutaneous tissue becomes thickened, the overlying skin becomes ulcerated, alopecic and punctuated with fistulae discharging a watery to oily exudate. Infection often tends to start in the inguinal region, although it can begin in the axillae, flanks or dorsum. The disease may subsequently spread to contiguous areas of the lateral and ventral abdominal wall, perineum and tail-base. Severely affected cats may become pyrexic, anorexic and reluctant to move, particularly if lesions are secondarily infected with skin commensals such as *Staphylococcus* and/or *Streptococcus* spp.

Occasionally, pyogranulomatous pneumonia caused by rapid-growing NTM has been reported in cats. These cases usually present with coughing, dyspnoea, fever, malaise, and weight loss. These infections have been caused by *M. fortuitum* in two cats (after aspiration of orally administered lactulose in one case)[93,133] and *M. thermoresistibile,* following a bath in another[102,134].

Uncommonly reported clinical presentations include peripheral vestibular disease, generalised involvement of lymphoid tissue, pulmonary disease, GI disease and intracranial infection causing subsequent neurological signs.

Infections caused by the fastidious NTM typically manifest as single or multiple cutaneous and/or subcutaneous nodules often located on the head, limbs or trunk, and regional lymph nodes may be involved[135,136]. As with the slow-growing NTM, nodules are typically non-painful and non-adherent to underlying tissues. The skin over the lesions may be intact, alopecic or ulcerated. Lesions caused by *M. lepraemurium* and *Candidatus* 'M. lepraefelis' tend to be found on the head, limbs and trunk (although anywhere on the integument can theoretically be involved), or may be widespread, involving many cutaneous sites[135]. Lesions caused by *Candidatus* 'M. tarwinense' are also mostly found on the head (particularly the eyes, lips or mouth and front limbs), and it has not been shown to cause widespread cutaneous disease[136]. Cases of *M. visibile* infection,[137] and at least one case of *Candidatus* 'M. lepraefelis',[8] have had systemic involvement confirmed at *post-mortem* examination.

1.2 Canine Mycobacteriosis

1.2.1 Tuberculosis

There is little published data regarding the incidence of tuberculous mycobacterial infections in dogs, and most of the information about this disease's clinical presentation comes from sporadic case reports, mostly of *M. tuberculosis*[138-143] and *M. bovis* infections[144-150]. There are only very rare individual cases of *M. microti* infection[151]. Anecdotally, the incidence of tuberculosis in the UK appears to be much lower in dogs than cats, but the clinical severity much greater. Dogs typically present with extensive GI disease (weight loss, vomiting, and diarrhoea) and pulmonary pathology (Figure 1.6). In the UK, dogs also seem more likely to become infected with MTBC pathogens than the other mycobacterial species, though this may be an artefact of which infections can definitely speciated with the limited availability of diagnostic tests for dogs, rather than a reflection of true incidence. As with cats, the range of clinical signs is wide and has been known to include reticulo-endothelial, musculoskeletal, cutaneous and/or neurological signs[146,147].

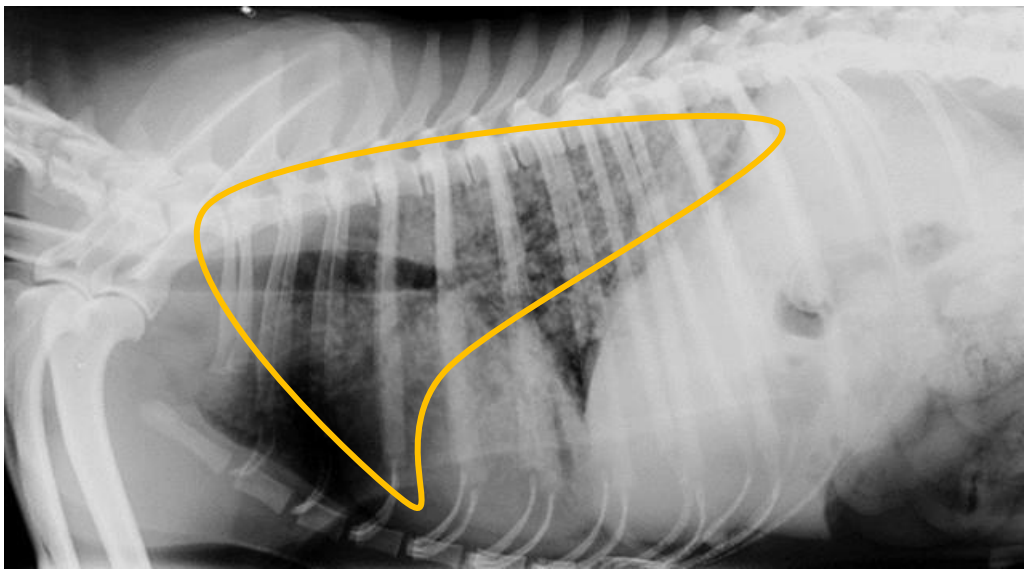

Figure 1.6: A latero-lateral thoracic radiograph of a six-year-old, male, neutered, Jack Russell terrier dog infected with *M. bovis.* The radiograph shows multifocal to coalescing areas of soft tissue opacity (granulomas; outlined in yellow) distributed throughout the lung fields with associated marked peri-bronchial lymphadenopathy.

The greatest risk factor reported for a dog acquiring an MTBC infection is for it to be cohabitant or in close contact with a human suffering from active tuberculous disease; most cases of *M. tuberculosis* infections in dogs have occurred because of reverse zoonotic transmission from an infected human *i.e.* anthropozoonosis[152,153].

1.2.2 Non-Tuberculous Mycobacteriosis

Slow-growing NTM

Typically, MAC organisms are the most frequent slow-growing NTM mycobacteria recovered from dogs[154-166]. Miniature Schnauzers and Basset Hounds are reportedly predisposed[154-166]. *M. avium* subspecies *avium, M. avium* subspecies paratuberculosis (MAP) and *M. avium* subspecies *hominisuis* have all been identified from canine samples[154-166].

Clinical presentations of canine MAC infection include multi-systemic disease, including any combination of generalised lymphadenopathy, anaemia, weight loss, hepatopathy and/or splenomegaly[154-166]. As with feline infections, many of these presentations are clinically indistinguishable from tuberculosis.

M. kansasii has been isolated from a three-year-old Whippet with chronic pleural effusion[167] and also from a mammary mass of a two-year-old Chihuahua[168]. The latter case also had chronic generalised demodicosis implying an underlying immune deficit was present, and it subsequently succumbed to the mycobacterial infection after prednisolone treatment for cutaneous lymphoma. Disseminated *M. genavense* infection was diagnosed in a two-year-old pug presenting with generalised lymphadenopathy, abdominal organomegaly, pyrexia and limb pain[169].

Localised dermal infections in dogs due to *M. ulcerans,* the causative agent of Buruli Ulcer, have been reported in endemic areas of Victoria, Australia[170]. This organism produced a cytotoxin, mycolactone, which is responsible for the deep skin ulcerations that are a hallmark of this disease. Although the exact ecology of *M. ulcerans* has not been determined, it has a worldwide but highly focal endemicity, with most human infections occurring in West Africa

and the coastal regions of South-eastern Australia. It is suspected that the organism gains entry via breaches in the epidermis, possibly in some cases involving mechanical insect vectors[171]. Animal cases have only been reported from Australia, and also include possums,[172,173] koalas,[174] a long-footed potoroo[172], alpacas[175], horses[176] and a cat[84].

Rapid-growing NTM

Sporadic canine infections have been reported. *M. goodii* was isolated from a dog with uncontrolled hyperadrenocorticism[177]. Systemic *M. smegmatis* group infection was diagnosed in a young Bassett Hound[178] (perhaps due to the same immunological defect that renders this breed susceptible to MAC infections). *M. fortuitum* group skin and pulmonary infections have also been reported. [179-183]

Fastidious NTM

Canine leproid granuloma (CLG) is caused by an, as-yet uncharacterised, fastidious mycobacterium related to *Candidatus* 'M. tarwinense' and *M. simiae*. It has greatest prevalence in Australia,[184] New Zealand,[185,186] Brazil[187-189] and the USA[190]. Zimbabwe (where the first case dates back to 1973[191]), and Colombia have also reported cases. In 2014, a case was diagnosed in Europe (Italy) for the first time[192] and cases have since been suspected in the UK (O'Halloran, *manuscript in preparation*).

Over-represented breeds include the Boxer dog and its crosses, Staffordshire bull terriers, Foxhounds and Doberman Pinchers. Short-coated hunting dogs, especially Spaniel breeds, have also been found to be predisposed and active hunting work has been shown to be a risk factor for the disease.[186,190]

The route of infection is proposed to be an arthropod vector due to the location of lesions which inoculates the mycobacteria into the skin and gives rise to the sites of disease and case predilection.

Clinically, the disease is characterized by cutaneous nodules on the head, especially involving the dorsal fold of the pinnae (Figure 1.7), but may also be located on the lateral trunk, rump

and/or limbs. Lesions may be singular or coalesce to cover large areas in some animals. As lesions get larger, they may ulcerate and can become pruritic if secondarily infected.[193] Affected dogs are typically systemically well and spread of the disease to draining lymph nodes, contiguous structures, as well as internal organs does not occur.

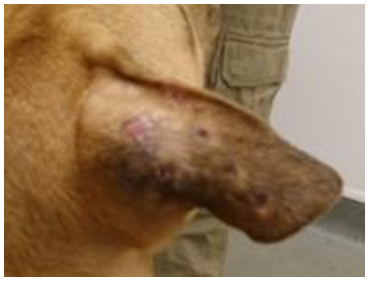

Figure 1.7: The first suspected case from the UK of 'canine leproid granuloma' lesions on the dorsal pinna of a three-year-old male neutered Labrador retriever dog. *Image courtesy of Fiona Fahy (MRCVS).*

1.3 Diagnosing Mycobacterial Disease in Companion Animals

A correct diagnosis of mycobacteriosis can be challenging to achieve, in companion animals as well as other species including humans and cattle. This is the case for a variety of reasons; importantly, several infectious agents can produce overlapping clinical signs whilst diagnostic tests can lack sensitivity.

Importantly, different mycobacterial species carry differing prognoses, optimal treatment choice, and zoonotic potential.

Feline cases that would warrant a high index of suspicion for mycobacterial infection include skin nodules or abscesses that do not heal and/or are only partially responsive to first-line antibiotic treatment such as amoxicillin. This is particularly true for geographical locations where there is traditionally a high prevalence of MTBC (e.g. UK) or feline leprosy infections (e.g. south eastern Australia, New Zealand).

Differential diagnoses of nodular lesions of the skin and subcutaneous tissues include infections due to other saprophytic bacterial species (such as *Nocardia spp.*), fungi and algae, eosinophilic granuloma complex, or primary or metastatic neoplasia.

1.3.1 Laboratory Abnormalities

During the clinical examination of any case of suspected mycobacterial infection, it is essential to fully establish the extent of any local disease and detect any cases with disseminated disease or systemic involvement.

Serum biochemistry and haematology

If abnormal, these typically reveal non-specific changes e.g. a stress leukogram. More significant abnormalities are usually reflective of more severe or systemic disease and include anaemia and an elevated serum calcium concentration.[194]

Hypercalcemia and hypovitaminosis D

Hypercalcemia has been described in human adults with most granulomatous disorders, including mycobacterial disease, although the majority of hypercalcemic patients are asymptomatic for their hypercalcemia. Reported rates of hypercalcemia in *M. tuberculosis*-infected (human) adults vary widely from 6% to 48%[195]. However, symptomatic hypercalcemia in human tuberculosis cases is uncommon, with rates of ~3% in adults[196]. The equivalent rate of hypercalcemia in companion animals in response to mycobacteria is unknown, but some cases have been reported,[24,39,194] whether this was causing or contributing to any of their clinical signs e.g. anorexia, is unclear.

Intimately linked with calcium metabolism is that of vitamin D. Briefly, vitamin D is hydroxylated in the liver to 25-hydroxyvitamin D (25(OH)D), the form most widely measured to assess vitamin D status, and is further metabolized by 1α-hydroxylase in the kidney to 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$; (calcitriol) which is the hormonally active form of vitamin D.[194-197] Calcitriol increases the concentration of calcium in the blood through increasing intestinal absorption, reducing renal excretion and inducing osteoclast-mediated reabsorption from bone.[194-197]

There is a long-standing association between vitamin D concentrations and mycobacterial immunity, and it has recently been shown that calcitriol has a directly anti-mycobacterial

activity *in vitro,* as macrophages are capable of producing calcitriol upon Toll-like receptor (TLR)-2 stimulation, when they phagocytose mycobacteria[198-200]. The result is that circulating 25(OH)D concentration decreases, and intracellular ionised calcium concentration increases. If the extent of infection is large, then these effects become detectable as systemic hypovitaminosis D and hypercalcemia.[198-200] This phenomenon has been long established in human medicine and more recently in feline patients with tuberculosis.[194,197] Similarly, in both species, the presence of either factor at diagnosis is a poor prognostic indicator. What remains unclear, however, is whether these findings are merely a marker of disease which has already disseminated (and so will inevitably be harder to treat), or whether they inherently influence disease outcome. Some clinical trials in humans have attempted to establish the benefit, if any, of supplementing tuberculosis patients with vitamin D during therapy as an alternative or in combination with reducing sub-clinical hypercalcemia[201-205], but the results to date have been discordant, and its use is not currently included in any treatment guidelines.

The exogenous retroviral status of cats should be established as these infections may be a poor prognostic indicator. That being said, FIV and/or FeLV-positivity has not been established as a predisposing factor for feline tuberculosis, unlike in humans where disseminated mycobacteriosis can be an "Acquired immunodeficiency syndrome-defining" infection,[206] and where Human Immunodieficiency virus (HIV) infection is a potent risk factor for tuberculosis and confers a particularly poor prognosis.[207]

1.3.2 Diagnostic Imaging

Radiography

Radiography is useful for detecting systemic involvement, especially pulmonary dissemination, and for monitoring disease progression and/or treatment response.[208] While radiographic changes of mycobacterial infection are variable, pathology is most frequently seen in the thorax, consisting typically of a diffuse interstitial, alveolar or bronchial pattern with peri-bronchial and sternal lymph node involvement observed with increasing disease severity.[208] It is important to note that no pattern is pathognomonic for mycobacteriosis (e.g. it

may also be consistent with a diagnosis of neoplasia or other infection e.g. toxoplasmosis) and that observed lung pathology can be mixed.

Computed tomography

Similar findings are seen with the use of computed tomography (CT) imaging, though the sensitivity of detection is increased with this modality, and a diffuse structured interstitial lung pattern is most common, being either nodular or reticulonodular in nature[209,210] (Figure 1.8). Both imaging modalities also play a key role in monitoring a patient's response to therapy (see *Prognosis*).

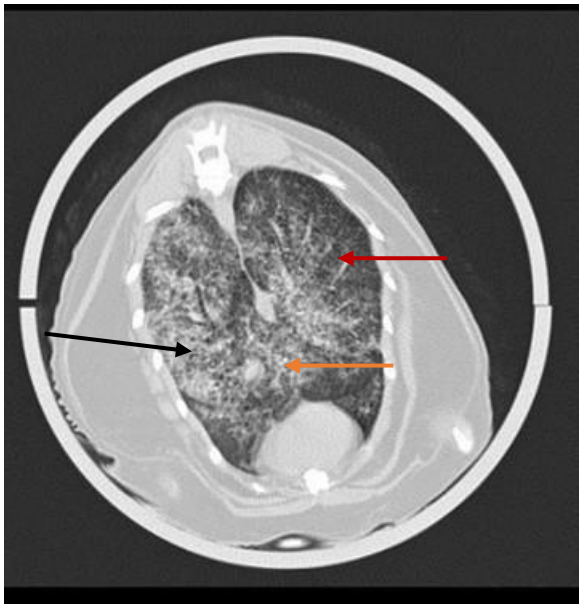

Figure 1.8: A transverse CT scanned section through the thorax of a three-year-old, male, neutered, retrovirus negative, Oriental cat caused by *M. microti* infection. The scan reveals a diffuse structured interstitial pattern (black arrow) comprising mixed nodular (orange arrow) and linear structures (red arrow), characteristic of a reticulonodular pattern which is typical of feline tuberculous pulmonary pathology. *Image courtesy of Alison Major (MRCVS).*

Abdominal imaging

This can be achieved using radiography, CT and/or ultrasonography, and can reveal hepatosplenomegaly, abdominal masses, mineralised or granulomatous mesenteric lymph nodes, and/or ascites.[23,24]

1.3.3 Specific Investigations

Cytology

Cytology can be very useful in the diagnosis of mycobacterial disease. Fine needle aspiration (FNA) of lesions can be used to prepare glass slides of sampled tissue. Subsequent Romanowski-type (e.g. Diff Quik™) staining may reveal granulomatous to pyogranulomatous inflammation with “negatively staining rods” or “ghost bacilli” either within the cytoplasm of reactive macrophages and giant cells, or within the extracellular space (Figure 1.9a). Subsequent slides are then frequently re-examined following acid fast-staining (e.g. using ZN or Fite’s method) to confirm the presence of mycobacteria. A modified ZN-staining procedure is occasionally needed for rapid-growing mycobacteria, as they are not as acid-fast as other species (Figure 1.9b). An FNA has the advantage of being less invasive than an incisional or excisional biopsy, but is likely to have lower sensitivity; particularly in tuberculosis lesions which frequently have few (if any) visible mycobacteria.

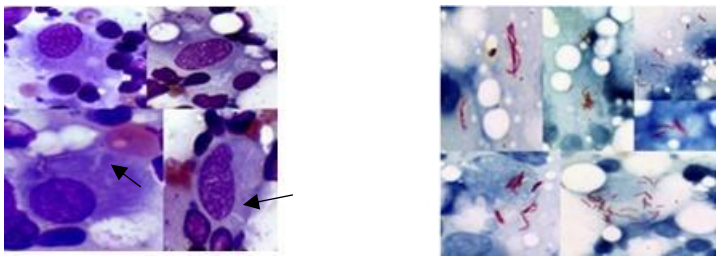

Figure 1.9a: *Left:* Cytological appearance of a FNA from a dermal granuloma in the skin of a three-year-old male neutered DSH cat due to *M. bovis* infection, stained with DiffQuick. Fields of view (x40 magnification) have been put together to display reactive epithelioid macrophages, which contain intracytoplasmic non-staining (“ghost”) bacilli consistent with the presence of mycobacteria (black arrows). *Right:* A Ziehl-Neelsen (ZN)-stained slide made from the same lesion taken at x100 magnification under oil emersion.

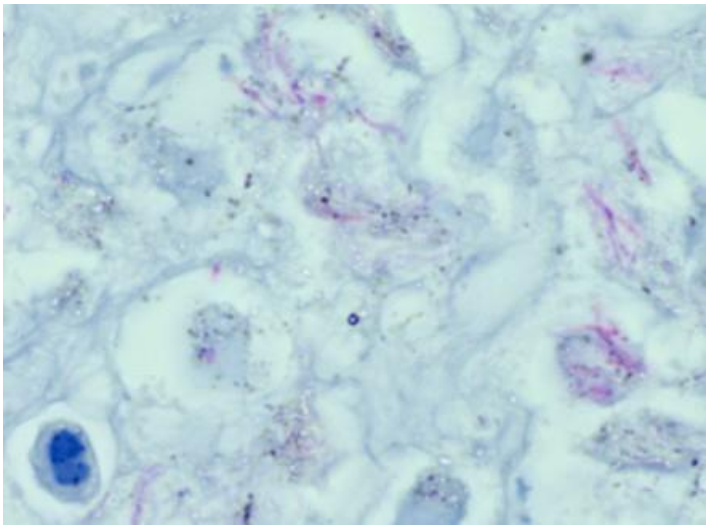

Figure 1.9b: A ZN-stained section (x100 magnification) of a granulomatous lesion biopsied from the nose of a one-year-old, male neutered, retrovirus negative, DSH cat caused by infection with *M. microti*. Staining reveals numerous positively stained (pink), slender, curved bacilli both intracellularly and within the extracellular space suggestive of mycobacteriosis.

Histopathology

Histopathology is frequently the first line of diagnostic investigation in these cases. The most typical approach taken is to surgically remove or biopsy a non-healing skin lesion, subcutaneous mass or chronically enlarged lymph node. In some cases, if there is only a single cutaneous lesion, this may prove curative.[211]

Histopathology alone cannot speciate mycobacteria, and this is needed to establish the risk to owners and other animals in the household, potential treatment options, and prognosis. However, typical histopathologic findings in most cats with mycobacteriosis include granulomatous inflammation with foamy, epithelioid and/or palisading macrophages, occasionally with significant infiltration of lymphocytes distributed at the periphery of the granuloma and/or neutrophils (*i.e.* pyogranulomatous inflammation; Figure 1.10).[211] ZN-staining may reveal highly variable numbers of AFB which is idiosyncratically influenced by the immune response of the host, the location of the granuloma, and the species of infecting organism (MTBC and rapid-growing NTM infections are generally considered have fewer visible AFB compared to slow-growing and fastidious NTM infections, though this is not always

the case, and is not diagnostic). The use of modified Fite's or fluorescent Auramine-O stain may increase the sensitivity of detection of mycobacteria in histological samples.[212]

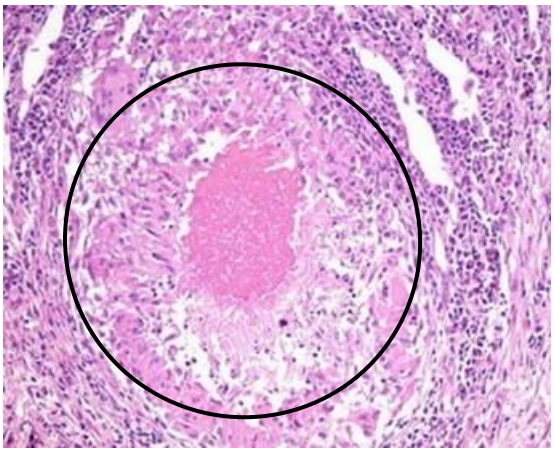

Figure 1.10: Histological appearance of granulomatous inflammation in a two-yeaar-old male neutered DSH cat caused by *M. bovis* infection demonstrating a typical focal, sub-dermal organising granuloma with a large area (outlined in black) of central necrosis. This section is stained with haematoxylin and eosin and imaged at x10 magnification. Higher magnification revealed extensive effacement of the normal tissue architecture with numerous palisading macrophages with large nuclei and foamy cytoplasm infiltrating the tissue surrounding the necrotic area. ZN-staining of the same biopsy (not shown) revealed moderate numbers of AFB within the area of necrosis. *Image courtesy of Shannon Street (MRCVS).*

Historically, the pathological picture seen with feline leprosy was divided into a tuberculoid form of disease which classically affected young cats with few mycobacteria being seen within the lesions, and a separate, leproid form which affected older cats, with numerous mycobacteria being seen within the lesions.[213,214] Greater insights have been gained from recent studies, and the pathology now appears to vary more with infective species of mycobacteria than patient demographics, with most infections caused by *Candidatus* 'M. tarwinense' and *Candidatus* 'M. lepraefelis' being lepromatous in nature (91-95%), whereas just over half (58%) of the *M. lepraemurium* cases were of this pathologic type in a recent study.[213,214]

Specialist Mycobacterial Culture

Isolation of mycobacteria following laboratory culture is currently the gold standard diagnostic test for confirmation of mycobacterial infection in UK companion animals[24]. However, in some laboratories it has a low sensitivity and may fail, even when AFB have been demonstrated in the lesion[24]. Ideally, samples should be sent to a recognised reference laboratory e.g. the Animal and Plant Health Agency (APHA) laboratory Weybridge in the UK. When culture is successful it can take a protracted period for some species to grow visible colonies, e.g. *M. microti* generally requires a minimum of 12 weeks to culture, during which time treatment needs to be instigated based on a presumptive diagnosis, especially if this is supported by previous histological evidence[24,211].

Drug susceptibility testing of rapid-growing NTM is not only useful for clinical purposes, but historically has also been used to provide phenotypic data for typing of isolates (for example, susceptibility to trimethoprim, polymyxin B, and tobramycin). The National Committee for Clinical Laboratory Standards Mycobacterial Subcommittee has nominated broth dilution as the gold standard,[5] although this can be technically demanding and is not offered routinely by many diagnostic laboratories.

Molecular Diagnostics

Polymerase chain reaction (PCR) testing provides a rapid and typically accurate diagnosis (excluding contamination) and can be performed on fresh, frozen or formalin-fixed-paraffin-embedded tissue and Romanowsky-stained (but not ZN stained) cytology slides.[215] There are currently no commercially available animal specific molecular-based diagnostic tests for the detection of mycobacterial species of veterinary importance. Available methodologies in the UK are those used for the diagnosis of active mycobacterial diseases in human patients. These tests have a high sensitivity for MTBC organisms *i.e.* those that are significant human pathogens, but are not validated for others that are uniquely of veterinary importance e.g. *M. lepraemurium.* The relative probability of successfully achieving a diagnosis from veterinary submissions is likely to be related to the number of AFB present in any given biopsy sample. Though exceptions have been documented[216], as a general rule, it is thought that samples

with moderate to large numbers of AFB are reasonably likely to yield a diagnostic quantity and quality of mycobacterial DNA; whereas those with few or no AFB are unlikely to provide useful results and other diagnostic methodologies are usually considered preferable in these instances.

There is not yet a consensus on a single gene that should be used for the genetic speciation of all mycobacteria. Even within the MTBC, the reductive evolution of these organisms complicates the picture. The most frequently utilized genes for mycobacterial identification are those which encode for; 16S rRNA,[215] the 65kDA heat shock protein (*hsp65*),[217] the β-subunit of bacterial RNA polymerase (*rpoB),*[218] 16-23S rRNA internal transcribed spacer ITS region[219,220] and, less frequently, the DNA gyrases (*gyr*). Assays targeting the insertion sequence (IS)6110 element for MTB complex,[221] IS2404 for *M. ulcerans,*[222] and multiplex IS901 and IS1245 for differentiation of *M. avium* subsp. *avium* and M. *avium* subsp. *hominissuis*[223] have also been utilised in the diagnosis of small animal infections.[53,66]

The MTBC mycobacteria have identical sequences at the 16S rRNA gene, and 16-23S internal transcribed spacer (ITS) region and all contain IS6110, therefore other genetic methods, including genomic deletion analysis,[224] spoligotyping[225] and mycobacterium interspersed repetitive unit and variable number of tandem repeats (MIRU-VNTR) analysis[226] must be used to differentiate the members of this group.

Although its popularity has reduced with the accessibility of genetic sequencing, PCR restriction fragment analysis or restriction fragment length polymorphism [RFLP] analysis is a commonly used DNA profiling technique for identification of NTMs. This technique involves amplification of a particular gene (e.g. ITS[227], *hsp65*[228,229], IS1245 for *M. avium*[230]), and subsequent restriction enzyme digestion, followed by analysis via agarose gel electrophoresis.

Matrix-assisted laser desorption ionization–time of flight mass spectrometry

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF-MS) can identify bacterial organisms quickly and cheaply. Although primarily used for cultured samples, it is now being used directly on clinical samples. Since the equipment used is relatively new, most veterinary laboratories have limited access to this technology; however, its use is likely to grow as the technology becomes cheaper and more available. The sample is mixed with an energy absorbing organic compound (known as 'matrix'). The resultant crystalized sample and matrix is then ionized by a laser beam. This results in the addition of protons to the liberated ions of the sample, after which they are then accelerated at a set potential towards a detector. The ions become separated according to the mass to charge ratio and the time it takes to reach the detector is known as the 'time of flight'. The resultant mass spectrometry produces a characteristic spectrum known as a peptide mass fingerprint. It is this fingerprint that is then compared to known results in a database, and the resultant organism can be identified. The available spectral database for mycobacteria is steadily improving. Mycobacteria require prior inactivation and disruption of the mycolic acid-rich cell wall, both for biosafety reasons (in the case of the MTBC) and to liberate proteins within the cells. Success rates vary but the differentiation between MBTC and NTM is usually very reliable.[231] MALDI-TOF MS requires more biomass compared to molecular techniques, which means substantially longer turnaround times for slow-growing mycobacteria.

Interferon Gamma Release Assay

The IFN-γ release assays (IGRA) were developed on the principle of quantitatively evaluating IFN-γ production by peripherally circulating antigen-specific effector T-memory cells upon *in vitro* stimulation with mycobacterial antigens, to aid the diagnosis of both active and latent tuberculosis in bovines, and subsequently adapted for human patients.[22] These assays have been widely adopted for the diagnosis of tuberculosis in cattle and humans in the form of the BOVIGAM™ and QuantiFERON-TB Gold test™ [203] respectively. In humans, the QuantiFERON test has been shown to have far greater sensitivity (80%) than the previously used tuberculin

skin test (also known as the Mantoux test; see later section) which has a reported sensitivity of just 28%[232].

The basis of IGRA tests is that either whole blood or peripherally-circulating blood mononuclear cells (PBMC) are incubated *in-vitro* in the presence of mycobacterial antigens restricted to specific subsets of mycobacterial species. The amount of IFN-γ produced is then quantified by enzyme linked immunosorbent assay (ELISA).[232-235] This value is then interpreted to support a diagnosis, in the context of clinical signs and other diagnostic investigations.[232-235]

The IGRA tests have several advantages over other diagnostic techniques for tuberculosis; they are significantly quicker at generating results than culture or even PCR methods, they are relatively non-invasive requiring only a single peripheral blood sample, and they can be repeated if necessary as conducting the assay does not alter the systemic immune response, unlike the tuberculin skin test[232-235].

Since 2008, an adapted IGRA has been validated for use in cats, and the QuantiFERON –TB Gold™ assay has been useful in assessing *M. tuberculosis* infections in dogs.[236-238] For the feline assay, PBMC are isolated from heparinised peripheral blood samples obtained from domestic cats with a high index of suspicion of mycobacterial disease e.g. they have clinical signs and/or histological findings indicative of mycobacterial disease[236,237]. These cells are then incubated for four days *in vitro* across several conditions;

a) complete cell culture media *i.e.* negative control,
b) a mitogen - a potent stimulator of IFN-γ production by all capable cells (*i.e.* a positive control),
c) purified protein derived from *M. avium*-complex (PPDA),
d) purified protein derived from *M. bovis* (PPDB) and
e) a combination of recombinant peptides 6kDa early-secreted antigenic target (ESAT-6) and 10kDa culture filtrate protein (CFP-10).

After four days, the amount of IFN-γ which has been released into the cell culture supernatant is measured by ELISA. A response to PPDB antigen which is greater than that to PPDA is indicative of MTBC infection with a sensitivity of up to 100% in cats.[236,237] The response to ESAT-6 and CFP-10 can be used to subdivide the MTBC as *M. microti* does not produce these molecules. Both ESAT-6 and CFP-10 are the major secreted antigens coded for at the *esx-1* locus on the region of difference (RD)-1 of the genome[239,240]. *M. microti* and *M. bovis*-BCG are RD-1 gene deletion strains as they do not possess the *esx-1* locus, but all other members of the MTBC do and so encode for these two proteins.[236-240] Therefore, in dogs the IFN-γ secretion levels that follow a pattern "PPDB > PPDA, and ESAT-6/CFP-10 positive", would be observed in response to either *M. tuberculosis* or *M. bovis* infection[238]. However, in cats this pattern is considered diagnostic of *M. bovis,* as this species is so rarely, if ever, infected with *M. tuberculosis*[236,237]*.* Unfortunately, some animals (bovine and feline) infected with *M. bovis* do not respond to the ESAT-6 and CFP-10 peptides by production of antigen-specific IFN-γ; a small study showed that this occurred in 20% of cats and a similar proportion (~18%) of cattle, so this test does not always reliably discriminate between *M. bovis* and *M. microti.*[236,237,240]

If the response to PPDA peptide is greater than PPDB (and ESAT-6/CFP-10 is negative), this is indicative of a MAC infection, while no response to any non-control peptides but where mycobacteria have been identified e.g. histologically, indicates an infection with another NTM organism[240].

Orthologues of the MTBC molecules ESAT-6 and CFP-10 are encoded by a small number of NTM (*M. kansasii*, *M. szulgai, M. marinum, M. riyadhense, M. genavense, M. bohemicum, M. interjectum, M. flavescens, M. xenopi, and M. malmoense*)[242] and so it is possible for patients to show a significant response to this protein combination only or in combination with PPDA as a result of infections with these organisms. Though no such results have been published, they have anecdotally been observed. Given that reports of domestic animals being infected with these species are limited to individual case reports[243,244], the likelihood of the IGRA false-diagnosing MTBC or MAC would seem to be low.

Screening in-contact animals

Where animals are known to be in epidemiologically significant contact with a patient that has clinically active disease but remains asymptomatic it is possible, unlike with all other testing strategies, to submit blood samples for IGRA testing. However, results obtained from such testing must be interpreted with caution. Large prospective cohort studies of human patients have shown that those with a positive tuberculin skin test (TST: see below) have a 1.4 to 1.7-fold higher rate of developing active tuberculosis within one year compared to those with a negative TST result.[245] Three studies provided incidence rate ratios of tuberculosis stratified by IGRA as well as TST status at baseline.[245] The association with subsequent incident tuberculosis in test-positive individuals compared to test-negatives was higher but not statistically significant leading to the conclusion by the World Health Organisation (WHO) that both IGRA and TST methods appear to have only modest predictive value, suboptimal sensitivity, and do not help identify those who were at highest risk of progression to disease.[245] Therefore, the decision of whether to test in-contact animals and then to instigate therapy or not based singularly on IGRA results is currently made at the discretion of individual clinicians and animal owners.

Tuberculin Skin Testing

The TST (also known as the Mantoux test, Mendel–Mantoux test, Mantoux screening test, tuberculin sensitivity test, Pirquet test, or PPD test) is a tool for screening human patients for tuberculosis.[245-250] The Mantoux method of the TST is the major tuberculin skin test used around the world, largely replacing multiple-puncture tests such as the Tine test. [245-250] The Heaf test, a form of Tine test, was used until 2005 in the UK, when it was replaced by the Mantoux method.[251] The TST is endorsed by the American Thoracic Society and Center for Disease Control and Prevention (CDC).[252,253] Purified protein derivative (PPD) tuberculin is a precipitate of species-non-specific molecules obtained from filtrates of sterilised, concentrated mycobacterial cultures.[248] The tuberculin reaction was first described in humans by Robert Koch in 1890 and the test was first developed and described by the German physician Felix Mendel in 1908.[245] A standard dose of five tuberculin units in 0.1 ml, according to the CDC,[253]

or two tuberculin units of Statens Serum Institute tuberculin in 0.1 ml solution, according to the National Health Service,[251] is injected intra-dermally and the test is 'read' 48 to 72 hours later[251-253]. A person who has been infected with mycobacteria will undergo a type IV (delayed) hypersensitivity reaction leading to a quantifiable swelling at the site of inoculation.[254] The reaction is read by measuring the diameter of induration (palpably raised, hardened area) across the forearm in millimeters.[251-254] The test is considered positive if it is greater than a pre-defined threshold which is altered depending on; the background risk or the patient, the clinical index of suspicion, the endemic prevalence of the country/region and any underlying immunological disorders.[251-254.]

In cattle; official *M. bovis* eradication campaigns also use intradermal tuberculin testing as statutory diagnostic assays for the detection of *M. bovis* specific cell-mediated immune responses developed during the infection.[255-257] Single intradermal skin testing (SIT) detects a delayed-type hypersensitivity reaction to the intradermal injection of PPDB, administered at the cervical site in Europe or at the caudal fold of the tail in USA and New Zealand.[255-257] Three days (72 +/- 4 hours) after inoculation, the skin fold thickness at the site of injection is examined and measured.[255-257]

The single intradermal comparative cervical tuberculin test (SICCT), *i.e.* comparison between PPDA and PPDB, is used in high prevalence areas of bovine *M. bovis* infections and allows better discrimination between animals infected with *M. bovis* and those infected with MAC or environmental mycobacteria which leads to improved test specificity.[257]

The use of tuberculin skin testing has been evaluated in both cats and dogs with mycobacterial infections and led to unreliable results which did not correlate with the findings of other established diagnostic tests and so is not used clinically.[238,259] Recent work in cattle has shown that performing the SIT/SICCT in ruminants can go on to boost both the cell-mediated and humoral immune responses of the individual animals leading to an increase in sensitivity for subsequently performed IGRA or antibody assays.[258] This effect has not been explored in companion animals to date.

1.4 Treatment of Mycobacterial Disease in Companion Animals

Once a diagnosis has been achieved (or while pending culture results but based on clinical signs and histopathological findings) the next challenge for clinicians and owners is managing a mycobacterial infection in a companion animal. If treatment is to be attempted, obtaining a successful outcome requires a prolonged course of multi-antibiotic therapy, and success is dramatically influenced both by owner and patient compliance. Drug toxicity, particularly in feline cases, and the costs associated with medications and disease monitoring can make optimal regimes difficult to maintain.

Drug-resistant tuberculosis with the detection of multi-drug, extensive-drug or total-drug resistant isolates, as well as the continuing human HIV pandemic, has dominated the focus of many of the recent changes to anti-mycobacterial drug therapies, including anti-tuberculosis therapy.[260-262] This is of even greater importance for drugs used in human protocols.

1.4.1 Treatment of Feline Tuberculosis

Surgery

As discussed, by far the most frequent clinical sign of feline tuberculosis is a single or small number of skin nodules.[23,24] In most cases, these are removed by excisional biopsy facilitating a diagnosis to be made. In these cases, complete surgical resection may be curative.[211] However, this cannot always be guaranteed, and adjunctive medical therapy is almost always indicated.[211] Surgery may be considered for any lesions remaining at the time of diagnosis or that may have subsequently arisen. However, there is a significant risk of wound dehiscence in many cases. There is no evidence that revision surgery confers a benefit to rigorous medical management,[211] so is not widely advocated.

In cases of intra-articular tuberculosis or significant osteomyelitis, medical management alone has consistently been found to be inadequate for successful management.[47,263,264] This is thought to be due to the lack of penetration of anti-tuberculosis medications into the synovial space, as well as the aggressive osteolysis caused by these pathogens, often before the time

of diagnosis (Figure 1.11). In such instances amputation of all or part (at least one joint above the lesion) of the limb with adjunctive medical therapy is often required for a successful outcome.[47]

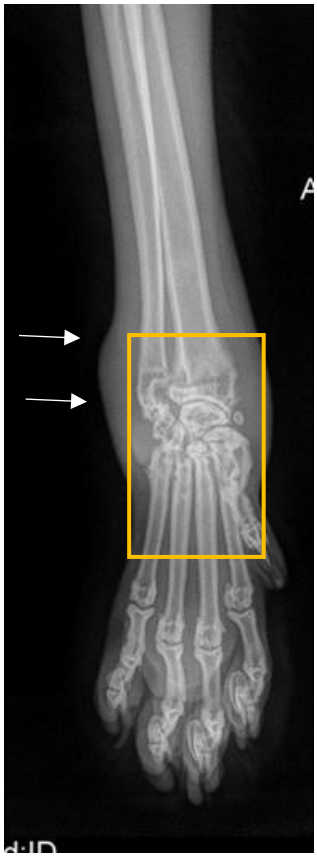

Figure 1.11: A cranio-caudal radiograph of the right distal forelimb of a four-year-old, male, neutered, retrovirus negative Maine Coon cat with an intra-articular infection with *M. microti.* The radiograph demonstrates severe osteolysis of the carpal bones as well as the distal radius and ulna (outlined in yellow) surrounded by extensive soft tissue swelling (white arrows). *Image courtesy of Professor Noel Fitzpatrick MRCVS.*

Medical therapy

The current CDC and WHO guidance on the appropriate drug combination for human treatment has changed and treatment with rifampicin, isoniazid, pyrazinamide and ethambutol (combined) for six months is now advocated.[265] This regime cannot be readily translated directly into veterinary medicine. Isoniazid and ethambutol have been associated with severe nephrotoxicity, hepatotoxicity, and neurotoxicity in companion animals, and isoniazid carries the further risk of inducing optic neuritis, as such these drugs should not be used as first-line therapies for companion animals.[266-270] Pyrazinamide is ineffective against *M. bovis*[271] and has

also been associated with hepatotoxicity in cats.[272] Given the lack of diagnostic capacity to rapidly and definitively diagnose the causative agent in companion animal mycobacterial disease, the use of pyrazinamide is currently considered contraindicated as a first line agent.

As an alternative regime, standard first line therapy for feline tuberculosis should comprise a fluoroquinolone, a macrolide/azalide and rifampicin.

The fluoroquinolone of choice is pradofloxacin (where available) due to its excellent safety profile in the cat and its known *in vitro* activity against mycobacteria when compared to older fluoroquinolones.[95] Where this is not available the use of moxifloxacin is advocated as it has a very similar pharmacological profile to pradofloxacin.[105] Alternative fluoroquinolones that could be considered would be the older drug marbofloxacin or the human drug ciprofloxacin (the active metabolite of enrofloxacin[273]). Enrofloxacin itself is an unacceptable choice in the cat due to its association with acute irreversible retinopathy.[274] The macrolide/azalide of choice is azithromycin due to a dosage pattern of once every 24 hours which eases adherence to treatment, plus its concentration within pulmonary macrophages.[275,276]

Rifampicin has long been a cornerstone of anti-tuberculosis, and more general anti-mycobacterial medical therapy. It belongs to the rifamycin class of antibiotics and acts to inhibit DNA-dependent RNA-polymerase activity in susceptible bacteria.[277] Importantly, it has good efficacy against both extracellular mycobacteria as well as those slowly and persistently replicating within macrophages following phagocytosis.[227] However, it should never be used as a monotherapy for mycobacterial disease as resistance develops rapidly.[278,279] Rifampicin is known to be a potent inducer of the hepatic cytochrome P450 system and so has the potential to be significantly hepatotoxic when used chronically.[280,281] Before starting treatment, it is advised that clinicians assess a patient's liver function by evaluation of serum biochemistry and haematology as well as considering bile acid stimulation testing if there are concerns. Treatment with rifampicin should be stopped if circulating levels of liver enzymes significantly exceed the upper reference interval, hyperbilirubinemia develops, or patients develop clinical signs attributable to hepatic dysfunction. Liver support drugs e.g. S-adenyl methionine (SAMe) have been used in a small number of cases both to prevent and reverse increases in liver

enzyme activity of feline patients on long term anti-mycobacterial therapy. Once recovered, the rifampicin can be re-introduced at half of the previous dose, while continuing with SAMe, plus close monitoring to ensure the hepatopathy does not recur. Rifampicin is excreted in all bodily fluids and may cause urine, tears, and saliva to discolour to red-orange in color.[282] This is not clinically significant, but it can be distressing for clients who are unprepared, especially as there is the potential for the discoloration to be mistaken for haemorrhage. Deposition of the drug within the skin can cause skin discoloration, irritation and, occasionally, hyperesthesia in cats; the complete converse of humans where it is used as a therapy for cholestatic pruritus.[283,284] Rifampicin is unevaluated a potential teratogen and so should only be handled by owners wearing gloves and should not be administered to pregnant queens.

Table 1.2: First-line anti-tuberculous medications for use in cats; a triple combination comprising one of the fluoroquinolones, a macrolide/azalide and rifampicin is frequently used.[285]

Drug	Product	Dosing	Contraindications	Side Effects
Pradofloxacin	Veraflox™ 25mg/ml suspension or 15mg tablets	5-7.5mg/kg PO q 24 hours	Not in dogs <12 months of age (18 months in giant breeds) or cats <6 weeks of age due to potential adverse effects on cartilage. Do not use in animals with epilepsy.	Neutropenia with high doses and/or long courses
Marbofloxacin	Marbocyl™ 20mg tablets	2mg/kg PO q 24 hours	As for pradofloxacin.	Intermittent vomiting and transient diarrhoea on instigation of treatment.
Moxifloxacin	Avelox™ 400mg tablets**	10mg/kg PO q 24 hours	As for pradofloxacin.	Occasional vomiting. Ocular toxicity has not been thoroughly assessed.
Ciprofloxacin		5-15mg/kg PO q 12 hours	As for pradofloxacin.	Occasional vomiting.
Azithromycin	Zithromax™ 250mg capsule	5-15mg/kg PO q 24 hours	Pre-existing cardiopathy esp. arrhythmogenic diseases.	Intermittent vomiting and diarrhoea possible but uncommon.

	50mg/ml suspension		Pre-existing hepatopathy.	
Clarithromycin	*Generic* 500mg tablet 50mg/ml suspension	7.5mg/kg PO q 12 hours	Concurrent use of NSAIDs or antacids. Pre-existing hepatopathy.	Pinnal erythema. Generalized erythema. Hepatotoxicity.
Rifampicin	Rifadin™ 300mg capsule 20mg/ml suspension	5-10 mg/kg PO q 24 hours by mouth	Pre-existing hepatopathy. Pregnant queens	Hepatotoxicity; induction of liver enzymes, anorexia, generalized erythema and pruritus. CNS[a] signs. Discoloration of body fluids.

PO; *per os* [a] Central nervous system [b] NSAIDs; non-steroidal anti-inflammatory drugs.

The duration of treatment is dependent on the clinical signs present at the time of diagnosis. Three months of triple therapy as outlined should be considered a minimum in all cases.[285] Treatment should be extended for two months beyond the resolution of all clinical signs which here also includes any pulmonary dissemination detectable on standard radiography (Figures 1.13a and 1.13b).[211,285] Treatment for cutaneous plus pulmonary disease is typically for six months. Monitoring for the resolution of pulmonary changes using CT studies is more difficult; due to the increased sensitivity of this modality, subtle changes such as minor scars remain detectable and may persist indefinitely beyond the need for treatment.[210]

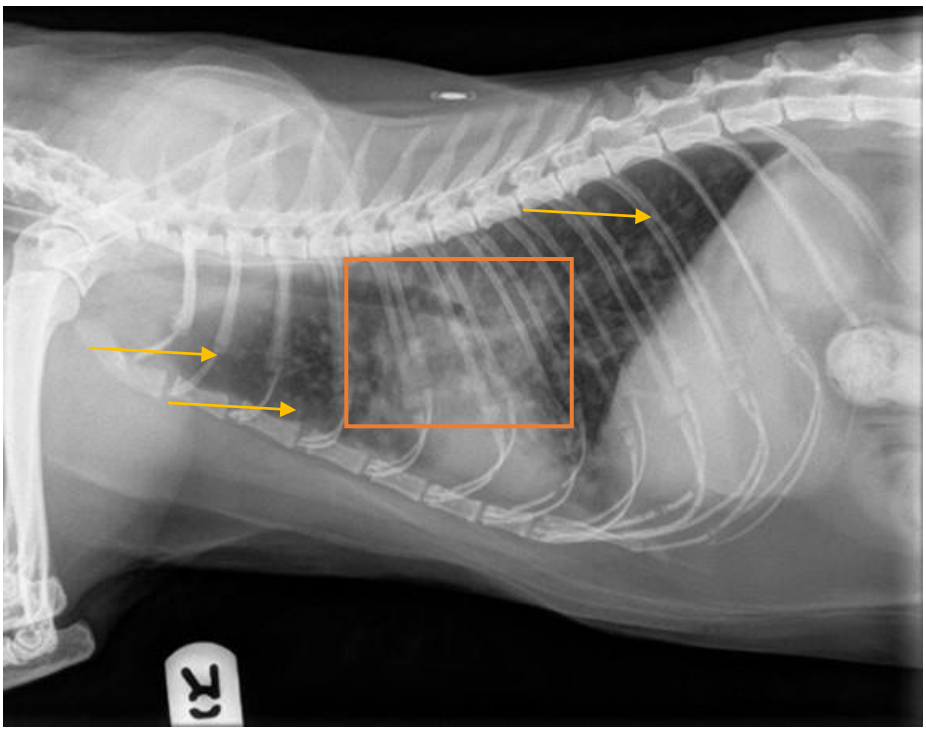

Figure 1.12a: A latero-lateral thoracic radiograph of a three-year-old, female, entire, retrovirus negative, DSH cat infected with *M. bovis.* The radiograph, taken at the time of diagnosis shows multifocal to coalescing areas of soft tissue opacity (granulomas) distributed throughout the lung fields (yellow arrows) with associated peri-bronchial lymph node enlargement (outlined in orange).

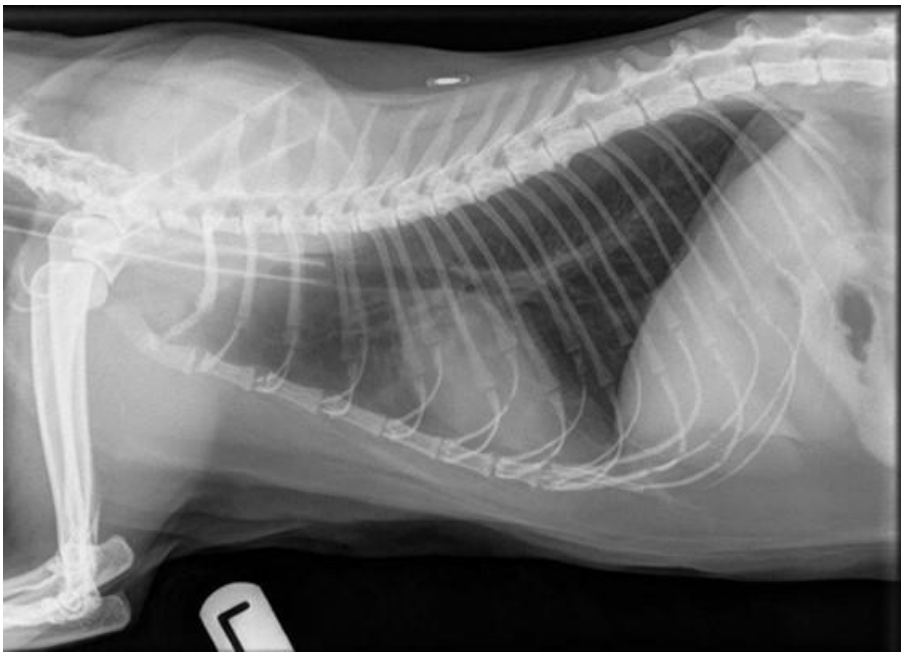

Figure 1.12b: A lateral thoracic radiograph taken from the same cat following three month of triple antibiosis consisting of rifampicin (10mg/kg), azithromycin (10mg/kg) and pradofloxacin (5mg/kg) given orally every 24 hours. The radiograph shows complete resolution of the previously identified pathology.

Therapy for refractory cases is more difficult as it requires the use of drugs which have increased frequency and severity of side effects. Rifampicin therapy should be maintained whenever possible and can be further combined with isoniazid and ethambutol.[211,285] Treatment should still last for a minimum of three months and two months beyond the resolution of clinical signs. [211,285]

Human patients who are believed to have latent infection (as they are IGRA positive for tuberculosis, but do not show any clinical signs), may be offered a six-month course of isoniazid monotherapy.[286] Currently there is no strong evidence to suggest that this reduces the incidence of those who go on to develop active disease in cats. However, such prophylactic therapy could be considered by individual clinicians on a case by case basis.

Table 1.3: Second line anti-tuberculous medications for use in cats; a triple combination should be used comprising both isoniazid, ethambutol and rifampicin (see Table 1.2).[285]

Drug	Dosing	Contraindications	Side Effects
Isoniazid	10-20 mg/kg PO q 24hours	Poorly evaluated in cats. Hepatic dysfunction. History of seizures.	Hepatotoxicity. Peripheral neuritis. Seizures. Acute kidney injury.
Ethambutol	10-25 mg/kg PO q 24hours	Poorly evaluated in cats.	Optic neuritis.

Prognosis

If dosing is maintained, then the prognosis is considered to be fair, with one review stating that 40% of cases reached and maintained complete clinical remission, though it remains unclear as to whether this constitutes bacteriological cure.[211] The remaining 60% of cases showed variable responses from temporary or partial remission to no response to treatment.[211] However, these data relate to a retrospective cohort, many of which were treated with what would be considered sub-optimal therapies, such as short courses of fluoroquinolone as monotherapy. With the approach outlined above, clinical experience suggests a positive prognosis of 70-80% of feline tuberculosis cases, particularly those with cutaneous and/or pulmonary involvement.[285]

1.4.2 Treating Feline NTM Infections

Surgical intervention is often a pre-requisite to obtaining a diagnosis in these cases. However, many feline NTM infections are progressive and are likely to recur following the excision of lesions, even when clear surgical margins have been achieved. It is, therefore, advisable to follow up surgical intervention with adjunctive medical treatment.

Slow-growing NTM

The treatment of some NTM infections (particularly *M. avium*) may be more difficult and less rewarding than for tuberculous infections. This is because many of these organisms do not readily grow in laboratory conditions, so it has not been possible to generate good sensitivity data across many clinical isolates from companion animals. Additionally, the drug resistance patterns of these organisms vary widely, not just between species but also between isolates of the same species.[287] Furthermore, *in vitro* drug susceptibility testing results do not always correlate with *in vitro* drug activity, as some of the genes which confer resistance (e.g. to macrolides) are inducible, so their presence does not necessarily correlate with observed susceptibility profiles, especially for MAC organisms.[287] The Clinical and Laboratory Standards Institute criteria for the antimicrobial susceptibility testing of NTM recommends broth

microdilution as the gold standard, preferably performed by a reference laboratory, or similarly experienced facility.[288]

Most published susceptibility data apply to members of the MAC complex, *M. kansasii* and rapid-growing mycobacterial species.[288] Recommendations for susceptibility testing of uncommonly isolated species, which have been assessed to be clinically significant in people, may include testing against rifampicin, clarithromycin, amikacin, ciprofloxacin, ethambutol, linezolid, moxifloxacin, and trimethoprim-sulfamethoxazole.[288]

The optimal drug regime is hard to readily determine. It is also important to note that MAC isolates are typically resistant to most commonly used second generation fluoroquinolones (e.g. enrofloxacin, marbofloxacin, orbifloxacin). Significant pharmacokinetic interactions have also been reported in human patients with NTM infections; e.g. rifampicin can reduce the bioavailability of both isoniazid and moxifloxacin, resulting in lower serum concentrations but extended half-life.[289,290] How far this contributes to the emergence of drug-resistant isolates and/or worse clinical outcomes is not clear and its implications for feline and canine patient treatment are currently unknown.

Anecdotally, the most successful protocols appear to consist of three drugs from; a macrolide (clarithroymycin or azithromycin), a fluoroquinolone (pradofloxacin, moxifloxacin or possibly marbofloxacin [in the case of isolates other than *M. avium*]), plus rifampicin or clofazimine. As with tuberculous infections, drug therapy should be continued for two to three months from the time of clinical remission to reduce the risk of recurrence. Some cats require extended (up to a year) or indefinite therapy to remain asymptomatic. Relapses have also been reported.[23]

Rapid-growing NTM

The management of mycobacterial panniculitis in cats is well described, particularly those caused by strains of *M. fortuitum* in the USA.[291-293] The use of lipophilic antimicrobial agents directed by susceptibility data and, where appropriate, surgical resection and reconstructive techniques has made this an eminently treatable disease in many instances.[291-293] All infections caused by rapid-growing NTM should undergo culture and drug susceptibility testing

but generally; members of the *M. smegmatis*-complex are usually susceptible to doxycycline and fluroquinolones but are often resistant to clarithromycin.[294,295] *M. fortuitum* tends to show higher levels of resistance to antimicrobials in general, although also tends to be susceptible to fluroquinolones and clarithromycin,[296,297] while *M. chelonae* tends to be resistant to all commonly used drugs except clarithromycin, gatifloxacin, and linezolid.[296-299] Minimum inhibitory concentrations for ciprofloxacin, enrofloxacin, moxifloxacin and pradofloxacin using the broth microdilution method have been reported for feline *M. fortuitum*, *M. goodii* and *M. smegmatis sensu stricto* isolates.[77,88]

In areas such as Australia, where *M. smegmatis* and *M. fortuitum*-complex infections predominate, treatment should commence empirically with one of the newer generation fluoroquinolones (pradofloxacin or moxifloxacin) [19,213,214], whereas in the USA where *M. chelonae* infections are more commonly diagnosed, the best first line choice is clarithromycin or azithromycin.[296] Multi-drug therapy is ideal to reduce to induction of resistant clones[297]. Thus, the addition of additional drugs (e.g. doxycycline) based on *in vitro* susceptibility data is recommended. In refractory cases, clofazimine, cefoxitin, linezolid or amikacin may be used if indicated by drug susceptibility data.[19,213,214]

Cases are reassessed every few weeks for evidence of adequate clinical response. In some cases, a cure may be achieved with medical therapy alone; however, some may eventually become refractory to treatment, requiring wide, sometimes radical, surgical *en bloc* resection of residual infection, and then continuation of appropriate antibiotics for a further few months.[130] The total duration of therapy for mycobacterial panniculitis caused by rapid-growing mycobacteria is usually three to 12 months (ideally, one to two months past clinical resolution of signs, as with other mycobacterial classes).[130]

Pneumonia caused by rapid-growing mycobacteria, depending on geographical location empirical treatment with pradofloxacin and/or clarithromycin should be started immediately upon the finding of mycobacteria within diagnostic specimens and then adjusted upon receipt of drug susceptibility data.[92,103] Treatment should always consist of using two or three suitable agents and should be continued for one to two months past resolution of signs.[285] Pulmonary

lesions that are refractory to appropriate medical therapy may need to be surgically resected, and antibiotic therapy should then be continued post-operatively for several months.

Fastidious NTM

There have been rare instances of spontaneous resolution of feline leprosy infections, especially those caused by *M. lepraemurium*,[300,301] although most cases require interventional treatment to achieve a cure. Definitive treatment guidelines for each of the fastidious species are yet to be established because the antibiotic susceptibility of these organisms is largely unknown due to the difficulty in culturing these organisms on cell-free media, and a lack of data from prospective treatment trials.

M. lepraemurium grown in cell-culture viability assays suggests that it is highly susceptible to rifampicin and clofazamine.[302] Although *M. lepraemurium* appears to be somewhat susceptible to dapsone,[302] the potential side effects of hepato- and neurotoxicity and blood dyscrasias limits the use of this drug use in cats.

Wide surgical resection of nodular skin lesions, plus clarithromycin combined with rifampicin or a newer generation fluoroquinolone, such as pradofloxacin or moxifloxacin (with or without clofazamine) appears to be the most efficacious treatment regimen (Table 1.4).[19,213,214] As with other mycobacterial infections, it is recommended that medical therapy be continued for at least two to three months past the resolution of clinical signs or following the resection of all visible lesions.

Table 1.4: Additional medications for the treatment of NTM infections of cats, in addition to those in Table 1.2.[19,213,214]

Drug	Dosing	Contraindications	Side Effects
Clofazimine	4-10mg/kg PO q 24 h *OR* 25-50mg/cat PO q 24 h	Poorly evaluated in cats	Hepatotoxicity. Gastrointestinal signs. Pitting corneal lesions. Photosensitisation.*
Doxycycline monohydrate	10mg/kg PO q 24 h	Dysphagia Concurrent barbiturate administration	Gastrointestinal signs; most frequently vomiting. Oesophagitis with the doxycycline hyclate salt of the drug
Amikacin	10-15mg/kg q 24 h by intravenous, intramuscular or subcutaneous injection	Poorly evaluated in cats	Nephrotoxicity. Ototoxicity.

*Due to the potential for photosensitization, cats should be kept indoors for the duration of treatment.

Prognosis

Data regarding the prognosis of feline NTM infections suggest variable success rates but resolution occurs in approximately 60% though the outcome of each case is likely multifactorial depending on several factors including the causative agent and the extent of the infection, with systemic disease carrying the worst prognosis.[19,213,214] Generally, localised infections caused by any species have a relatively favourable prognosis if treated with an appropriate combination of drugs and surgery, if necessary. Given the nature of the often expensive and time-consuming schedule of multidrug therapy for many months many treatment failures result from lack of compliance.

1.4.3 Treating Canine Tuberculosis

The basis of therapy for canine MTBC infections is almost identical to that developed for feline infections. It consists predominately of medical therapy comprised of triple combination antibiosis. As for cats, the preferred combination of rifampicin, a macrolide/azalide and a fluoroquinolone (ideally pradofloxacin) should be administered for a minimum of three months and for at least two months beyond clinical resolution. In the dog, in addition to the fluroquinolones described for use in cats, enrofloxacin is a possible treatment option (Table 1.5). Because dogs are *generally* more compliant with the oral administration of medications clarithromycin is the preferred choice of macrolide due to its proven *in vitro* anti-mycobacterial efficacy.[296] It is also less expensive than azithromycin (because it is available as a generic product in the UK) which may be an important consideration when treating large breed dogs.

Zoonotic transmission of MTBC agents from dogs to their owners has historically been thought most likely when the dog has cavitating lung granulomas (tubercles) and a productive cough resulting in the expectoration of organisms which can aerosolise and then be inhaled by humans in close contact with the patient.[41,146,259,304] There has been recent advocacy towards the inclusion of antitussive agents such as codeine phosphate into the treatment of dogs that are actively coughing in order to reduce the zoonotic risk.

Table 1.5: Data for the use of enrofloxacin in the dog.[216]

Drug	Product	Dosing	Contraindications	Side Effects
Enrofloxacin	Baytril™	5-20 mg/kg PO q 24 h	Not in dogs <12 months of age (18 months in giant breeds) due to potential adverse effects on cartilage. Do not use in animals with epilepsy.	Gastrointestinal signs.

The prognosis for canine tuberculosis is considered guarded to poor. This is likely a reflection of the advanced degree of systemic disease and consequent multiple organ dysfunction that is typically present at the time of diagnosis. It may also reflect an increase in virulence associated with *M. tuberculosis* infection which is relatively common among MTBC infected dogs. It is possible, however, that if *M. bovis* or *M. microti* infections were recognised early and appropriate therapy instigated, then similar outcomes as those seen for feline tuberculosis could be achieved.

1.4.4 Treating Canine NTM Infections

There is a paucity of canine-specific data due to the rarity of infections, but successful outcomes have been achieved following the same therapeutic protocols as those used for feline NTM infections. One dog was successfully treated for disseminated *M. genavense* with clarithromycin, ethambutol, and enrofloxacin.[169]

Cases of CLG typically resolve spontaneously within one to three months of diagnosis.[184,189,303] The efficacy of reported treatments is difficult to establish; however, occasionally persistent infections of greater than three to six months are observed, and in these instances treatment

with anti-mycobacterial agents is probably warranted. Where lesions are localised, minimal surgical resection and then adjunctive medical therapy is frequently curative. Combination antibiotics, known to have efficacy against the slow-growing mycobacteria are appropriate choices, for example, pradofloxacin/moxifloxacin, rifampicin, clofazimine and/or clarithromycin. Some authors have reported successful use of topical clofazimine in silver sulphasalazine, with or without dimethyl sulfoxide.[303]

Treatment of *M. ulcerans* infection in dogs typically involves eight to ten weeks of a third-generation fluoroquinolone (e.g. enrofloxacin) plus either clarithromycin or rifampicin.[170] Occasionally, surgical debridement is helpful, particularly for recalcitrant lesions.

1.5 Public Health Risks of Companion Animal Mycobacteriosis

Before beginning treatment, it is important to ensure that owners and persons in close contact with the patient are fully informed of, and clearly understand the potential zoonotic risks associated with being in contact with an infected animal. The greatest risk is posed by members of the MTBC of mycobacteria:

- ***M. tuberculosis:*** Though rare, infection of any companion animal with *M. tuberculosis* would be considered a significant zoonotic risk.[39,41,138,139-143] Finding an infected companion animal should trigger a search for a possible infecting human. Infected companion animals should be euthanased and their bodies cremated (not buried).
- ***M. bovis:*** Currently, only ~1% of human tuberculosis cases in the UK are caused by *M. bovis* infection.[11] Globally, in the last 150 years, only six cases of human *M. bovis*-tuberculosis have been published that resulted from exposure to cats, and where infections have occurred the cats have had skin lesions discharging purulent material containing many AFB.[285] Since 2014, Public Health England, Public Health Wales and Health Protection Scotland have all considered the risk to humans from *M. bovis* infected pets to be "very low".[305] That said, the risk is still present and should be considered seriously in the context of humans with specific risk factors for transmission (see below) and the clinical signs present. Extensive and/or purulent lesions pose the greatest risk to human health and are generally less responsive to treatment. By comparison, single non-ulcerated skin lesions and/or regional lymphadenopathy carry very low zoonotic risk and may be very amenable to treatment.[285]
- ***M. microti:*** The risk to humans of *M. microti* is significantly lower than that of *M. bovis*.[306] Fewer than 30 human cases of *M. microti* infection have been documented in published literature and 11 (~40%) of these had specific risk factors (see below) – none have been shown to have resulted from exposure to an infected cat or dog.[306]
- **MAC:** Whilst not members of the MTBC, this group of organisms can infect humans in the presence of specific risk factors (see below) – again, none have been shown to have resulted from exposure to an infected companion animal.[307]

1.5.1 Specific Risk Factors for Zoonotic Transmission

This list has been compiled from advice published by public health organisations from across public health services across the UK[304,305], the CDC[252] as well as WHO guidelines[245]. Humans are considered at heightened risk if they:

- are under five years old (some sources suggest 12 years)
- are pregnant
- are HIV-positive
- suffer from substance misuse
- have been diagnosed with diabetes mellitus
- suffer (severe) kidney disease
- have ever received a solid organ transplant
- are a cancer patient receiving chemotherapy or radiation therapy
- have any medical condition requiring treatment with systemic corticosteroids
- require specialized treatment for rheumatoid arthritis or Crohn's disease
- are receiving tumour necrosis factor alpha (TNF-α) inhibitors
- have undergone gastrectomy or jejunoileal bypass
- suffer from silicosis

In any of the above situations, owners and veterinarians are strongly discouraged away from animal treatment.

1.6 The Immune Response to Mycobacterial Infection

The innate immune system is a crucial component of the early response to mycobacterial infections.[308-314] Myeloid cells of the innate system initiate antimicrobial pathways early and throughout infection which are key drivers that can act to limit clinical disease, but conversely these cells can also act as persistence niches for mycobacteria. [308-316] Established doctrine dictates that, ultimately, protective immunity against mycobacterial infection/disease lies within the development of an effective adaptive immune response, and a strong $CD4^+$ αβ T-cell response in particular, forming classical adaptive cell-mediated immunity (CMI) within infected hosts. [313,314,317,318.]

Cellular immunity is frequently defined as the protective mechanisms of the immune response other than antibody production, usually mediated by T lymphocytes, which is readily induced in response to intracellular pathogens.[313] Whilst the innate and adaptive responses are often examined in isolation it is far more probable that *in vivo* these systems act contemporaneously and synergistically.[311-313.]

1.6.1 Cells of the Monocyte-Macrophage Lineage

Many immune cell phenotypes are involved in the response to challenge, infection, control and potential elimination of mycobacteria once an individual has been exposed including macrophages, classical dendritic cells (cDC), both $CD4^+$ and $CD8^+$ $CD3^+$αβ T-cells, γδ T-cells, B cells and natural killer (NK) cells.[307-315]

Of all these cells, arguably one of the most critical is the macrophage, as their maturation and activation at the site of infection dominates the early stages of the innate immune response against mycobacteria.[311-314] Cells of the monocyte-macrophage lineage (MML) originate from bone marrow and precursors enter peripheral circulation from where they egress into tissues and become mature phagocytes.[315,319-322] Committed progenitor cells within the MML progress through a series of well-defined and morphologically distinct stages; a common myeloid progenitor shared with granulocytes gives rise to monoblasts, pro-monocytes and then

monocytes which migrate into the blood and extravasate into tissues where they are particularly associated with mucosal surfaces. [315,319-322] Similarly, cDC form a phagocytic network protecting mucosal and external body surfaces.[323,324]

During this differentiation process, macrophages acquire the surface expression of proteins which aid the functionality and identification of cells from the lineage.[323-326] Such immunophenotypic markers include, but are not limited to, cluster of differentiation (CD)14, CD172a (also referred to as SIRPα), CD16, CD163 and ADGRE1 (also referred to as F4/80).[320, 323-326]

The production of mononuclear phagocytes from progenitor cells is directed by colony-stimulating factors, which are to some extent lineage restricted and hierarchical in their actions.[319-322] These include macrophage colony-stimulating factor (CSF-1, also known as M-CSF), IL-34 (an alternative ligand of the CSF-1 receptor [CD115]), granulocyte-macrophage colony-stimulating factor (GM-CSF, also known as CSF-2) and fms-like tyrosine kinase 3 ligand (Flt3L).[315-326] These growth factors instruct the common myeloid progenitor to adopt a mononuclear phagocyte differentiation fate.[319-322]

The biological roles of cells from the MML are complex compared to other mature phagocytic cell phenotypes; for example, compared to neutrophilic granulocytes they possess marked longevity, play a pivotal role in embryonic development and in *post-natal* life are critical to wound and tissue repair.[315-326] However, the defining functional features of mononuclear phagoctyes include; the ability to sense and migrate towards gradients of microbial products and/or signalling molecules such as certain chemokines, phagocytose large particles such as whole microbes (e.g., bacteria, fungi) or dying cells, present antigens to the adaptive immune system, secrete chemokine and cytokine mediators resulting in the migration and activation of immune and non-immune cells and they exhibit cytotoxic activity against neoplastic or senescent cells. [315-326]

Whilst the majority of scientific literature regarding these cells focusses on murine and human models, previous work has shown that mature macrophages can be generated from both

circulating feline and canine monocytes (monocyte derived macrophages, MDM) cultured *in vitro* in the presence of the recombinant human (rh)-CSF-1 protein.[321,325,327-329] These cells were defined as being macrophage-like based on their *in vitro* morphology and surface expression of molecules including CD14 and major histocompatibility complex clas II (MHC class II) for feline cells, and CD163 on canine cells. [327-329]

Similarly, bone marrow cells cultured in the presence of rh-CSF-1 or recombinant CSF-2 has been shown to reliably generate mature macrophages (bone marrow derived macrophages; BMDM) in a number of species including sheep,[330] cattle,[331,332] goats, buffalo, pigs[320,334] and horses,[333] although, to date, not companion animals or humans.[334] These studies have all shown that these cells fulfil the earlier functional definition of mononuclear phagocytes.[330-334] The generation of cDC *in vivo* is complex but it is widely accepted that it is Flt3L-dependent.[335-337] *In vitro,* along with recombinant CSF-2, the terminal differentiation of dendritic cells *in vitro* from $CD14^+$ monocytes also requires the presence of the cytokine IL-4.[338,339]

Monocytes, macrophages, and cDC express a large number of cell surface proteins that mediate functional roles.[315,340-343] Microbial pattern recognition receptors (PRRs) are an essential component of innate immunity, they detect and recognise conserved pathogen associated molecular patterns (PAMPS), resulting in the activation of stimulated MML cells (and neutrophils) as part of the host response to eradicate invading pathogens whilst maintaining immunological tolerance by recognition of self.[311-316] An important class of PRRs is the TLR family which recognise a wide range of microbial pathogens and pathogen-related products.[311-316] TLRs are expressed to a far higher degree by monocytes than neutrophils.[315,316] Upon binding of specific ligands, TLRs signal via a pathway involving the adaptor protein MyD88, or via a MyD88-independent pathway involving TIR-domain-containing adapter-inducing interferon-β (TRIF), to activate NF-κβ and stimulate pro-inflammatory cytokine production from a range of cells including monocytes and macrophages.[311-316] Other cell-membrane associated receptors cooperate with specific TLRs to enhance pathogen recognition, for example, CD14 and MD-2 bind lipopolysaccharide (LPS)

and interact with TLR-4 to facilitate recognition and enhance eradication of Gram-negative bacilli from circulation and infected tissue sites.[344,345]

In response to mycobacteria, tissue resident macrophages become classically activated, in murine studies referred to as acquisition of the M1 phenotype, and produce a plethora of pro-inflammatory cytokines including TNF-α, IL-1β, IL-6 and IL-10.[346-348] Although the mechanisms of activation and TLR agonism appear to be remarkably conserved across mammalian species, the results of this activation can lead to differing outcomes across the variety of species for which this has been studied.[332-334] The TLR4 interaction with its co-receptor MD-2, CD14 and LPS is perhaps the best-characterised of these activation pathways.[344,345] A large body of evidence suggests that TLR-4 agonism induces TNF-α expression by macrophages from a wide range of species.[315,328,329,339,348] In contrast however, whereas mouse and chicken BMDM respond to LPS stimulation by upregulating arginine metabolism, inducible nitric oxide synthetase (iNOS) and nitric oxide synthase 2 (NOS2) to produce free radical 'reactive nitrogen species' (RNS);[334,349] stimulated BMDM/MDM from rabbits, sheep, goats, monkeys, horses, and badgers do not[349-356]. Previous evaluation of bovine alveolar macrophages has conflictingly shown that these cells, unlike BMDM derived from this species, are able to induce NOS2 mRNA and produce nitric oxide (NO) in response to LPS.[356-359] It is not currently known if this reflects differences in study protocols or functional and/or true metabolic differences between the cell types of possibly differing maturation states and disparate locations. Recently published comparative genome analysis has hypothesised that the species differences may be explained by variations in distant upstream gene promoters which are relatively poorly conserved.[356]

There are also pathogen associated differences, for example *M. tuberculosis* does not induce NO production from human MDM *in vitro* whilst *M. avium* induces a significantly greater NO response than uninfected control cells.[360] This may be part of the explanation for the host restriction of mycobacteria so that some species such as humans can be persistently infected with *M. tuberculosis* without always developing clinical disease.

Overall, however, the relative role that alternatively activated metabolic pathways may have in contributing to the comparative susceptibility or resistance of an individual or species to mycobacterial infection or disease remains untested. Whilst a theoretical contribution has been argued for in the literature,[354] no link has yet been proven and to date there is no experimental evidence to definitively support the hypothesis that macrophages which do not produce NO are more permissive to mycobacterial replication or less competent at mycobacterial killing than those that do.

1.6.2 The Initiation of Infection

Infection with *Mycobacteria* spp. begins once the physical barriers to infection are breached.[361-368] Primarily in humans, cattle and in occasional companion animal cases, tuberculous infections occur via the respiratory tract.[285,361-368] Small aerosols consisting of droplet nuclei which contain an infectious dose of MTBC mycobacteria are expectorated by an individual animal (of the same or different species) with active pulmonary disease; this dose is considered to be as few as one to ten viable colony forming units (CFU) for most species.[361-368] Due to the small size of the droplets they can remain aerosolised for extended periods of time, and are subsequently inhaled by a susceptible individual. [361-368]

Also by virtue of their small size, these inhaled particles are able to avoid the physical bronchial defence mechanisms and penetrate into the terminal alveolar space whereupon the mycobacteria present are recognised and engulfed by phagocytes; predominantly alveolar macrophages and cDC. [361-368] Mycobacteria may also interact with non-phagocytic cells in the alveolar space including alveolar endothelial cells and both Type 1 and Type 2 pneumocytes.[361-368] The interaction between parenchymal cells and infectious organisms is thought to be enhanced by the presence of IFN-γ, which is itself produced in response to such an infectious challenge.[369]

In cases where the respiratory tract is not the primary site of infection, as occurs in the majority of feline mycobacteriosis cases, the skin is breached e.g. by a bite from an infected rodent, leading to direct intradermal inoculation of the bacteria.[23,24,285] This almost certainly then allows

them to be directly exposed to resident (*i.e.* mature) tissue macrophages and cDC, as occurs with intradermal vaccination in other species.[370,371] This interaction is known to be extremely rapid; studies of intradermal vaccination of neonatal calves with *M. bovis*-BCG demonstrated trafficking of mycobacteria-laden antigen presenting cells to the local lymph node within half an hour of inoculation and MAP has been shown to translocate across the bovine gut with similarly rapid kinetics.[372-376]

In gastrointestinal cases of mycobacterial disease (Section 1.1.1), the bacteria are understood to be phagocytosed by gut-associated macrophages.[373-376] Macrophages and antigen presenting microfold cells of the gastrointestinal mucosa play a unique role in host immunity and tolerance.[377,378] These cells reside predominately within the lamina propria (with fewer present deeper within the muscularis and sub-muscosal gut layers) and gut-associated lymphoid tissues such as the Peyer's patches.[375,376] Microfold cells exist within close proximity to vast numbers of commensal bacteria and under normal healthy state conditions murine cells are known to constitutively possess an anti-inflammatory gene expression profile and transcriptome, for example producing IL-10 and transforming growth factor (TGF)-β.[379] Holding this state is believed to be essential for maintaining tolerance to commensal bacteria and food antigens and can become dysregulated in disease states such as inflammatory bowel disease (IBD).[377-380]

The mechanism of invasion of the alimentary system by mycobacteria in companion animals has yet to be investigated; however, the example of the comparatively well studied bovine model of paratuberculosis (Johne's disease) may provide some insights into the mechanisms involved in other species. Johne's disease in ruminants is caused by MAP which breaches the host gastrointestinal defences to establish a chronic infection.[375,376] The preferred route of entry for MAP is thought to be direct microfold cell invasion mediated by the formation of a fibronectin bridge between MAP organisms and microfold cell surface integrins.[381] The mycobacteria then traffic rapidly to the mesenteric lymph nodes where they establish persistence niches, in part by preventing the formation/maturation of the phagolysosome.[382] A supplementary cellular invasion mechanism for MAP is direct entry into epithelial cells via the

apical surface, whereupon an as yet ill-defined phenotypic change in the mycobacteria facilitate increased direct invasion of both other epithelial cells and gut-associated MML cells.[375]

Studies of white-tailed deer (*Odocoileus virginianus*) naturally infected with *M. bovis* have identified a high frequency of lesions (over 76% of infected animals in one study) with lesions in the palatine tonsils.[383,384] Similarly in cattle a lower but still significant proportion (40%) of skin test positive cattle naturally infected with *M. bovis* had lesions within the palatine tonsils.[385,386] This suggests that the tonsils may play an important role in the pathogenesis of tuberculosis in ruminants and possibly in other species too.[383-387] The tonsils are ideally located to sample bacteria entering through the oral or nasal cavities; mycobacteria may become trapped within tonsillar crypts, where follicle-associated epithelia actively take up microorganisms via microfold cells, a process which may have a significant impact on the efficacy of oral mycobacterial vaccines.[386-390]

It is possible that the same or similar mechanisms to these may allow NTM and MTBC organisms to invade the alimentary system of companion animals and cause clinical disease.

1.6.3 Phagocytosis and Antigen Presentation

Phagocytosis of pathogens, apoptotic cells and debris is a key feature of MML cell function in host defence and tissue homeostasis.[315,316,319,321,323] Phagocytosis is a process by which macrophages and other phagocytic cells take up relatively large, extracellular particulate material, such as bacteria and senescent cells, degrade them in a controlled manner and subsequently present antigen epitopes to T-cells. [313,315,316,319,321,323]

The agonism of PRR on MML cell surfaces induces a cascade of signalling events; the phosphorylation of multiple tyrosine kinases, cellular membrane extension, pseudopodia formation, actin polymerisation, reorganisation of the actin-based cytoskeleton, formation of a cell membrane bounded vesicle enclosing the phagocytosed particle and its engulfment into the cytoplasm to generate the structure of the early phagosome which continues to develop

and mature.[391-400] Phagocytosis of mycobacteria can occur through a diverse range of mechanisms, such as opsonic phagocytosis whereby foreign material is coated in proteins present in serum, namely complement proteins and antibodies, which subsequently bind to specific receptors on the surface of phagocytic cells *i.e.* complement receptors or immunoglobulin-G constant region receptors (FcγR1 [CD64], FcγR2 [CD32] and FcγR3 [CD16]).[391-400] In addition, non-opsonic phagocytosis can occur when phagocytes recognise and directly bind PAMPs on the surface of pathogens via receptors such as CD14 and CD163.[391-400]

Phagocytosis is a major target of mycobacterial immune evasion (Section 1.6.5), but in instances were phagosome maturation is completed, these organelles fuse with lysosomes which carry an arsenal of hydrolytic enzymes and anti-microbial peptides to form the phagolysosome.[391-400] The acidic and highly oxidative environment of the phagolysosome is essential for the activation of cathepsins, oxidants and cationic peptides with the goal of eventually leading to pathogen lysis and clearance.[391-400]

A further essential downstream function of phagocytosis is the presentation of antigen to $CD3^{+}\alpha\beta$ T-cells (Figure 1.13).[401-416] This process requires pathogen components generated by phagolysosome-mediated lysis to be loaded onto major histocompatibility complex proteins, to be trafficked through the Golgi apparatus to the cell surface where the antigen epitope is presented to the T-cell receptor of naïve cells.[401-416] Mature, tissue resident cells of the MML (*i.e.* macrophages and cDC) possess all three of the MHC subtypes; class I , class II and class III.[401-416] The mononuclear phagocytes predominantly present antigen in association with MHC class II molecules.[401-416] In addition B-lymphocytes (also referred to as B-cells) are also professional antigen presenting cells despite their absence from mucosal surfaces and their inability to phagocytose.[401-416] Instead the uptake of antigen is via receptor-mediated endocytosis.[401-416] The B-cell receptor has uniquely high affinity for a given antigen and the processing of antigen onto MHC class II is therefore extremely efficient, even when the quantity of antigen is low. [401-416] This interaction in shown in Figure 1.13 and the role of B-cells in mycobacterial infections is discussed in Section 1.6.4.

Classically, antigen derived from phagocytosis or endocytosis becomes surface-associated with MHC class II and is then presented singularly to naïve $CD4^+CD3^+$ T-cells whilst cytosol derived antigens are presented on MHC class I to $CD8^+CD3^+$ T-cells.[401-416] However, cDC have enhanced antigen presentation capacity compared to macrophages and will readily "cross present" phagocytosed antigen epitopes to both $CD3^+$ T-cell subsets.[401-416] This is shown schematically in Figure 1.13.

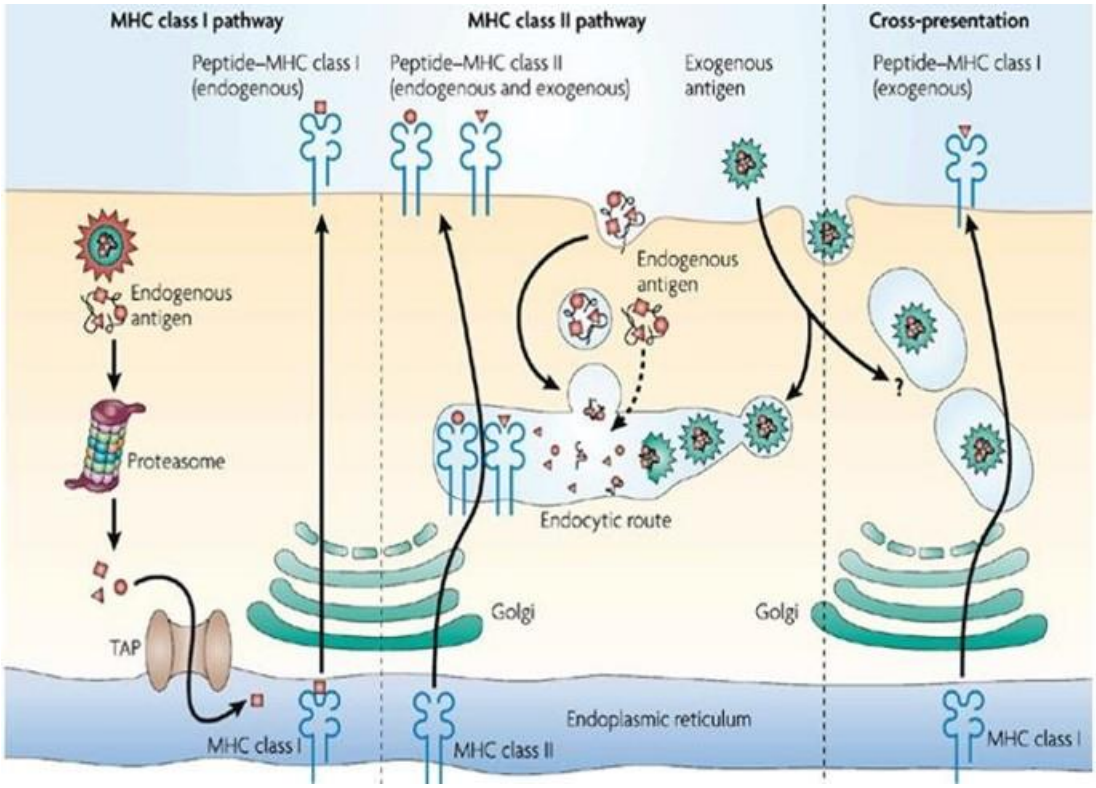

Figure 1.13: A schematic representation of antigen presentation pathways utilised by the professional antigen presenting cell phenotypes: macrophages, classical dendritic cells and B-lymphocytes to naïve $CD4^+$ and $CD8^+$ $CD3^+$ αβ-T-cells subsets via MHC classes I and II. Reproduced from Lybaert *et al.* (2017) In view of personalized immune-therapy. *Advanced Science,* **4(6):**1-8.

1.6.4 The Role of B-cells in Mycobacterial Infection

The intracellular nature of mycobacterial pathogenesis results in the development of a strong CMI response with the production of antibodies historically only thought to occur in the late stages of disease progression.[417-421] Activated B-cells, defined (in mice) by surface CD19 expression, and plasma cells, defined (in mice) by surface CD138 expression, are generally

considered to play a supportive role in the response to MTBC organisms as opposed to an essential or critical one.[417-421] However, B-cell aggregates have been demonstrated in proximity to MTBC mycobacteria in infected mice, cattle, humans and non-human primates.[422,423] The role of these co-localised B-cells may include enhancement of the CMI response by providing an alternative mechanism for antigen presentation, antibody dependent cytotoxicity and/or activation of the classical complement cascade which requires antibody-antigen complex formation.[417-423] For example, the opsonisation of *M. tuberculosis* with antibodies present in autologous heat-inactivated sera has been shown to enhance phagocytosis, intracellular mycobacterial killing and pro-inflammatory cytokine production by macrophages,[424] suggesting that there may be an enhancement of the immune response against mycobacteria by the activation of B-cells, and possibly for the mycobacteria of the MTBC in particular.

Though considered to be a proportionately minor part of the adaptive immune response to MTBC, the presence of specific antibodies in circulation provide an attractive option for the development of novel diagnostic tests.[257,430] Serodiagnostic assays by their nature are comparatively cheaper and quicker than tests relying on the detection of delayed hypersensitivity (*i.e.* CMI) responses to inoculated mycobacterial antigens (tuberculin, or PPD) such as the human TST or bovine SICCT[430] which are described in Section 1.1.3. Antibody-based tests are additionally beneficial as they can be used to test an individual animal at a single time point,[257] which is particularly useful for wildlife reservoirs of infection such as the Eurasian badger (*Meles meles*) in the UK[425,426] and white tailed deer in the USA compared to tuberculin testing which require the test to be "read" after a set time interval.[427-429]

Early investigations into the utility of serodiagnostics for the detection of bovine *M. bovis* infections initially utilised relatively crude mycobacterial lysates such as PPDB but found these assays to have poor specificities, presumably at least in part due to the large number of shared antigens between MTBC and environmental mycobacteria, to which many cattle are likely sensitised in the course of grazing pasture.[430] The elucidation of (bovine) immunodominant antigens within PPDB which are specific to *M. bovis* (and other MTBC mycobacteria) such as

MPB70 (a soluble secreted protein), MPB83 (a glycosylated lipoprotein), ESAT-6 and CFP-10 have greatly improved the specificity of these assays.[431-433] A number of such testing protocols and products are now commercially available and these have reported specificities of up to 98%.[431-433]

The IDEXX *M. bovis* Ab assay (IDEXX Laboratories, USA) is a conventional antibody capture ELISA based system which detects circulating antibodies against MPB70 and MPB83.[434,435] This system can be automated, providing the capacity for whole herd screening programmes which are both rapid and economical.[434] Waters et al. (2011) evaluated this assay using sera collected from naturally infected cattle from four countries; the USA (n=122), the UK (n=188), Eire (n=130) and New Zealand (n=42).[434] The sensitivity varied greatly by country with as high as 77% reported for UK cattle but only 40% for New Zealand cattle.[434] It is probable that the drop in sensitivity is a reflection of the lower prevalence of infection in New Zealand (and the USA) compared to the UK as this, by definition, has a profound impact on the sensitivity and positive predictive value of any diagnostic test. Alternatively, it may reflect differing stages of disease with more advanced disease (such as a lesioned phenotype) increasing the circulating antibody titres and therefore sensitivity of this and other serodiagnostic assays.

Enfer Scientific (Eire) have developed a multiplex fluorescence immunoassay which utilises antigen arrays to detect antibodies specific against up to seven *M. bovis* antigen preparations in several species, including cattle, goats, banded mongooses (*Mungos mungo*) and alpacas (*Vicugna pacos*).[435-440] The sensitivity and specificity obtained in cattle with this immunoassay has varied with the population under study.[435-437] In goats confirmed as *M. bovis* infected by SICTT, histopathology and culture, the multiplex test detected 57/60 (95.0%) of positive animals in one herd and 120/120 (100%) in a second herd and gave positive signals in a further 4% of SICTT-negative animals.[439,441] It has been reported as having a lower sensitivity in the UK for detecting *M. bovis* infections in cattle when compared to the BOVIGAM™ IGRA (by approximately 10%) but critically it has the capacity to differentiate between *M. bovis* infected and *M. bovis*-BCG vaccinated calves,[436,437] making it potentially useful as a DIVA assay (discrimination between infected and vaccinated animals) which is an essential pre-

requisite to the widespread introduction to cattle herds of *M. bovis*-BCG as a vaccine against bovine tuberculosis in the UK.

Chembio Diagnostic Systems (USA) have developed rapid animal-side testing platforms including two dual path platform (DPP) assays; the VetTB Assay for Cervids and the VetTB assay for Elephants which detect *M. bovis* and *M. tuberculosis* infections respectively.[442-444] The DPP technology involves two nitrocellulose strips which are connected in a "T" shape inside a cassette device (Figure 1.14). This allows independent delivery of the test sample and the antibody-detecting reagent, in contrast to the single-strip format used previously e.g. in the CervidTB STAT-PAK test.[430] The DPP VetTB test requires as little as 5µL of serum sample and provides a cage side result that can be obtained within approximately 20 minutes.[442] The presence and intensity of the visible line produced in response to either of the two separate antigens used, MPB83 and a CFP10/ESAT-6 fusion protein can be assessed.[442]

Figure 1.14: The VetTB Assay for Cervids. The sample well (top left) and the buffer well (bottom centre) form a 'T-shape' nitrocellulose strip. A positive antibody response leads to the development of a visible line within the result window (top right) after approximately 20 minutes incubation at room temperature.

1.6.5 Mycobacterial Avoidance of Host Immunity

Following phagocytosis, rather than initiating the mechanisms described above for pathogen degradation and subsequent antigen presentation, mycobacteria have evolved mechanisms to evade immune mediated destruction.[446-449] This may lead to intracellular replication within MML cells, and for mycobacteria to be able to survive for an extended period prior to the development of an effective CMI response.[446-449] Development of CMI has been shown to take approximately two to eight weeks to reach (diagnostically) detectable levels in humans following *M. tuberculosis* infection[235] and approximately four weeks following experimental bovine infection with *M. bovis*.[445] This delay in the induction of CMI may occur in part due to virulent mycobacteria possessing a multitude of evasion tactics which aim to subvert the early phases of the host innate immune response and thus allow mycobacterial replication and the establishment of infection. They inhibit host defence mechanisms such as phagosome maturation, phagolysosome fusion, antigen processing and presentation as well as IFN-γ signalling.[446-449] These mycobacteria may also escape the phagosome into the cell cytoplasm whereupon they reinstate or maintain an environment conducive to their own survival and replication by scavenging toxic oxygen radicals and RNS produced by the host to kill them.[446-449]

The RNS, are toxic to mycobacteria and are produced by macrophages via calcium-insensitive iNOS upregulation.[198,396] MTBC organisms can avoid host-mediated killing by preventing the fusion of RNI-containing lysosomes with phagosomes and by directly detoxifying RNIs.[446] For example the PPE2 protein encoded by *M. tuberculosis* travels to the host nucleus and inhibits NO formation.[4501,451] At least a further 30 genes are upregulated in MTBC mycobacteria when cultured in the presence of RNS including two haemoglobin-like molecules which may act as “nitrogen sinks”, demonstrating that these pathogens have evolved a significant number of mechanisms to avoid host mediated destruction by this mechanism.[450,451] During this process mycobacteria become established within the protected niche of the innate immune cells, where they can persist, potentially indefinitely.

As discussed in Section 1.6.1, the recognition of highly conserved PAMPS by specific PRR is a pivotal early stage in the co-ordination of the host immune response against mycobacteria.[346] Cellular components of mycobacteria are recognised by host receptors including TLR-2 and TLR-4, nucleotide-binding oligomerisation domain (NOD)-like receptors, dendritic cell-specific intercellular adhesion molecule grabbing non-integrin (DC-SIGN), dectin-1 and C-type lectins including CD206 (the mannose receptor).[344,345,452] These receptors may be expressed by both immune and non-immune cells e.g. fibroblasts and epithelial cells.[452] An intracellular signalling cascade is initiated following PAMP recognition by TLR which leads to altered gene expression downstream through transcriptional regulation.[452] Binding to other receptors such as DC-SIGN, CD206 and complement receptors may provide alternative routes of MML cell entry for mycobacterial species; for example *M. tuberculosis* has been shown to specifically target DC-SIGN on human pulmonary cDC (but not alveolar macrophages) as a mechanism of entry, following which, further cDC maturation is impaired reducing IL-12 secretion whilst they are induced to produce IL-10 and so act to supress the activation of other local immune cell phenotypes.[453,454]

Stimulation of the TLR-2 and TLR-4 receptors as well as the TLR-1 and TLR-6 receptors by mycobacterial derived ligands results in downstream activation of the nuclear transcription factor NF-κβ which in turn leads to the up-regulation of genes encoding pro-inflammatory cytokines TNF-α, IL-1β and IL-6. [344,345] The expression and secretion of a range of chemokines, reactive oxygen and nitrogen species including NO is also induced. The pro-inflammatory response is regulated by a negative feedback mechanism in order to prevent host tissue damage from a cytokine storm or systemic inflammatory response syndrome (SIRS).[455-457] This feedback is mediated by a family of tyrosine kinases (Tyro3/Axl/Mer aka TAM) which restrict TLR mediated signalling.[455-457] This endogenous negative feedback is exploited by the MTBC. Mycobacteria directly agonise TLR-2 for prolonged periods, resulting in endogenous downregulation of MHC class II in a subset of macrophages, reducing their ability to present antigens.[455-457] It has been suggested that this subset of macrophages cannot, therefore, subsequently prime naïve T-cells and so provide a niche for mycobacteria to persist out with host recognition.[455-457]

The evasion of macrophage-mediated destruction allows mycobacteria to replicate within the phagosome and induce necrosis of the engulfing cell. The released bacilli can continue to replicate extracellularly and are subsequently taken up by surrounding phagocytes. Whilst macrophages continue to fail at halting mycobacterial replication, cDC migrate to the local lymph node and prime $CD3^+$ T-cells (both $CD4^+$and $CD8^+$ subtypes) against mycobacterial antigens. As discussed above, the subversion mechanisms mycobacteria enact differ between cDC and macrophages, the persistence mechanisms which target cDC in particular may facilitate wider dissemination of infection via lymphatic drainage whereas intra-macrophage persistence would seem more likely to allow local infection to establish at the initial point of challenge. The delay in infected cells reaching lymphoid centres has the effect of delaying the instigation of the adaptive immune response and therefore allows mycobacterial persistence.

Apoptosis, an alternate mechanism of cell death, can also be manipulated by mycobacteria. It has been shown that there is increased $CD4^+$ and γδ T-cell apoptosis when cells from (human) tuberculosis patients are cultured with *M. tuberculosis* antigens compared to those from healthy controls.[458-461] Similarly, pulmonary tuberculosis is associated with increased soluble FAS ligand concentrations, decreased lymphocyte proliferative responses, reduced IL-2 secretion and IL-2 receptor expression whilst T-cell apoptosis also occurs in areas of caseous necrosis within tuberculous granulomas.[458-461]

1.6.6 Granuloma Formation

The hallmark lesion of mycobacterial infections, including tuberculosis, is the granuloma; a compact, organised aggregate of immune cells formed as part of the host immune response to the persistent stimuli of pathogens.[462-464] The formation and structure of mycobacterial granulomas have been studied in significant detail in recent years with the development of a zebrafish (*Danio rerio*) model using infection with *M. marinum.*[465] The most impressive feature of this model is the ability to perform non-invasive, high-resolution, long-term time-lapse and time-course experiments to visualise infection dynamics with fluorescent markers due to the transparent nature of the host.[465] This, coupled with the ease of genetic manipulation since

the advent of CRISPR-Cas9 technology, has allowed studies to be performed which had previously been impossible in other model species. For example, one recent study compared the time taken to achieve granuloma formation, or lack of it, in zebrafish deficient in the *RAG1* gene which is essential for the development of an adaptive lymphocyte immune response.[466,467] In-depth characterisation of bovine and human granulomas formed during *M. bovis* and *M. tuberculosis* infections respectively has shown a remarkable level of cross-species conservation of the key features of granuloma formation.[468-470]

Immune cell types present within a granuloma include macrophages, cDC, neutrophils, αβ T-cell subtypes, γδ T-cells, NK cells and reactive stromal cells; all of these cells produce a complex array of cytokines and chemokines which act to drive granuloma development (Figure 1.15).[468-470] As discussed above, following phagocytosis, mycobacteria-induced inhibition of phagosome-lysosome fusion enables the intracellular survival and replication of pathogen within macrophages which rupture, releasing mycobacteria into the extracellular space leading to the local recruitment of macrophages and dendritic cells.

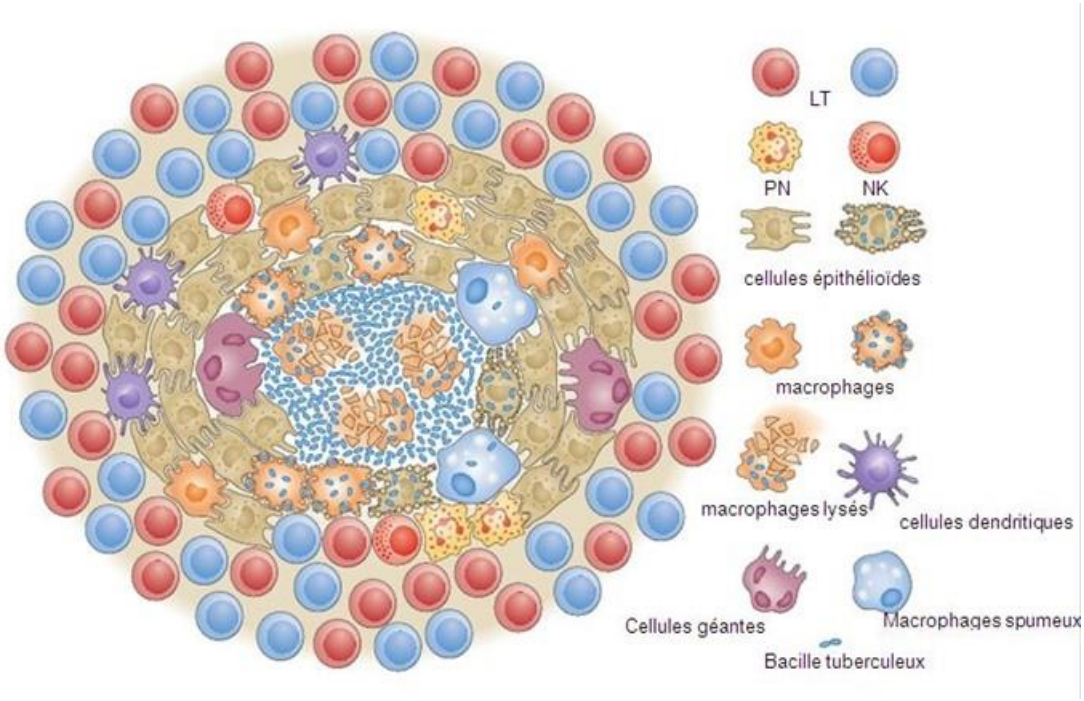

Figure 1.15: Structure and cellular constituents of the tuberculous granuloma. Reproduced from Ramakrishnan *et al.* Revisiting the role of the granuloma in tuberculosis *Nature Reviews Immunology* 2012;(12):352–366.

Within a granuloma, some macrophages undergo transformation to have tightly interdigitated cell membranes that link adjacent cells; these epithelioid cells can be highly phagocytic but in some cases do not contain bacteria at all. [468-470] Granuloma-associated macrophages can also fuse into multinucleated giant cells or differentiate further into foam cells, which are characterised by lipid accumulation that givens them their foamy appearence.[468-470] Foam cells have been noted to be most frequently located at the rim of the necrotic centre of a mature tuberculous granuloma. The consequences of these changes are not well understood, but in general, foam cells and multinucleated giant cells have been reported to contain only a few mycobcateria, if any. Mycobacteria are most commonly present in the central necrotic areas in which dead and dying macrophages can be seen.[468-470]

Mycobacteria laden cDC migrate via lymphatics to the local lymph nodes and present antigen via MHC class I and upregulated surface MHC class II to naïve T-cells resulting in priming of both $CD4^+$ and $CD8^+$ subsets.[468-470] The primed antigen specific T-cells migrate back to the focus of infection under the influence of chemokines such as the CXCL11-CXCR3 axis.[468-470] Subsequently, an accumulation of activated, occasionally necrotic, macrophages and mature cDC exist in the centre of the granuloma surrounded by tightly adhered (palisading) macrophages which develop an epithelioid phenotype.[468-470] Surrounding this are activated fibroblasts which being to lay down a fibrous capsule which is then, in turn, ringed by primed T-cells, giving rise to the formation of a “classical” granuloma at the site of infection as is frequently seen in human and bovine tuberculosis infections (Figure 1.15).[468-470]

Within a granuloma, the secretion of cytokines is a key driver of continued control of infection and maintenance of latency.[446,447] One the most important is the production of IFN-γ by $CD4^+$ T-cells, NK and γδ T-cells.[445,471,472] IFN-γ has been shown to activate macrophages, inducing increased synthesis and secretion of cytokines, particularly TNF-α, the production of both nitrogen and oxygen radicals and the enhancement of phagolysosome fusion.[445]

The γδ T-cells are a unique population of lymphocytes, like other non-conventional T-cell subsets bearing invariant T-cell receptors (TCRs), such as CD1d-restricted NK cells, γδ T-cells exhibit several characteristics that place them at the border between the innate immune

system.[471,472] The γδ T-cells may be considered a component of adaptive immunity in that they rearrange T-cell receptor genes to produce functional diversity and will develop a memory phenotype. However, the various subsets may also be considered part of the innate immune response where a restricted T-cell receptor may be used as a PRR.[471-475] For example, according to this paradigm, large numbers of (human) Vγ9/Vδ2 T cells respond within hours to mycobacteria, and highly restricted intraepithelial Vδ1 T cells will respond to stressed epithelial cells bearing sentinels of danger. Recent work has shown that human Vγ9/Vδ2 T cells are also capable of phagocytosis, a function previously thought to be exclusive to cells of the MML.[481,482] In cattle, a broadly equivalent subset are the γδ T-cells bearing the workshop cluster (WC)1 which accumulate rapidly at the site of infection. A number of bovine studies have shown that recruitment of γδ T-cells leads to the rapid expansion of the local population at the site of infection within six hours of intradermal inoculation (for example with PPDB or *M. bovis*-BCG).[471-475] The recruited cells infiltrate the granuloma and *in vitro* studies using bovine $WC1^+$ γδ T-cells has demonstrated their significant capacity for the secretion of IFN-γ and other chemokines which may aid the development of an appropriately organised granuloma.[471-475] Very little is known about γδ T-cells of companion animals, beyond rare case reports of lymphomas caused by their neoplastic replication.[476-480]

The exact relationship between the granuloma and a host's attempt at mycobacterial clearance is still not yet fully understood. The current paradigm asserts that the granuloma acts to physically wall off bacilli and limit further dissemination of infection into local tissues whilst simultaneously producing an ideal microenvironment for the interaction of different immune cells and the cytokines that they produce.[313,364,369] At the same time, the hypoxic, acidic environment created at the centre of the granuloma induces the expression of a number of genes within the mycobacteria such as *plcA, PlcB* and *PhoP* which are associated with the induction of a state of dormancy and replication cycle arrest.[483] This complex, ongoing process of interactions between the innate and adaptive immune system and mycobacteria is thought to be the means by which human hosts achieve latency.[446]

Studies have shown that only approximately 5-10% of individuals infected with *M. tuberculosis* will succumb to clinical disease within the first two years after infection (termed “primary tuberculosis”), a further 5-10% will develop disease later in life (termed “post-primary tuberculosis”), whilst the remainder develop life-long latent infection with no signs of disease at any time.[245,265] In companion animals, the relative frequency of the different possible clinical outcomes of infection *i.e.* sterilising immunity, primary or post-primary disease, or latency, is unknown. It may also be significantly different when infection is caused by different members of the MTBC or NTM species.

Recrudescence of infection into post-primary disease is most frequently believed to result following a local or systemic decline in the hosts immune response and therefore capacity to dynamically contain mycobacteria with granulomas, examples of which include the occurrence of an immunosuppressive/modulatory myeloid disorder e.g. lymphoma, prolonged periods of stress, the development of chronic kidney disease or diabetes mellitus, or old age.[245,265,446] In companion animals, many of these conditions occur frequently within the aged population, and in cats in particular, so the possibility of recrudescence of any mycobacterial infections must be considered to be of potential clinical significance.

1.7 Aims and Objectives

The aim of this project is to improve our current understanding of mycobacterial infections in companion animal species, and tuberculosis in particular.

In order to achieve this aim, this project will meet the following objectives:

1. Improve on the diagnostic tests currently available for cats and dogs by developing a molecular assay for formalin-fixed paraffine embedded (FFPE) tissue, which can identify the presence of mycobacterial DNA in tissue samples and discriminate members of the TB complex.
2. Use samples acquired clinical cases and from outbreak situations to identify the potential diagnostic methods for canine mycobacterial diseases given the paucity of currently available options
3. Examine the feline and canine immune responses to mycobacteria in depth to increase our understanding and to identify future diagnostic candidates.
4. Develop an *in vitro* model that can be used to futher study the early-host pathogen interaction between companion animals and mycobacteria in order to allow future research that may develop novel diagnostic or therapeutic intervensions.

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Chapter 2: Development of a PCR Assay for the Detection of *Mycobacteria spp.* in Companion Animal Formalin Fixed Paraffin Embedded Tissue Biopsies.

Abstract

Molecular methods are increasingly being used for the diagnosis of mycobacterial infections in human patients but no validated molecular testing methods are currently commercially available for the assessment of mycobacteria present in feline or canine tissue samples in the UK. This study is the first to develop a polymerase chain reaction assay for the speciation of mycobacterial infections that focusses on the UK companion animal population. In line with molecular techniques described to diagnose lepromatous diseases in both cats and dogs, this study extracted amplifiable DNA from the majority of formalin-fixed paraffin-embedded tissue samples despite most of them containing few acid-fast organisms when Ziehl–Neelsen stained sections were graded using a newly developed bacillary grading system. The six-gene mycobacterial panel targeting the *hsp65*, 16s rRNA, Ag85-B, Rv1506c, ESAT-6 and IS-1311 genes was validated using cultured reference isolates of mycobacteria. Subsequently, the assay was used to examine 380 feline formalin-fixed paraffin-embedded tissues and eight canine samples where mycobacterial infection was suspected. Importantly, no cases of *M. tuberculosis* infection were identified. In the feline tissues the prevalence of *M. bovis* infections was found to be 11.3%, broadly similar to previous analysis of mycobacterial culture results whilst the prevalence of *M. microti* (30.6%) was significantly higher in this study population than has previously been reported. *M. avium-intracellulare*-complex infections were only rarely identified in this cohort of feline samples. Sequencing of the *hsp65* gene fragment diagnosed nine feline infections caused by *M. malmoense* (n=3), *M. smegmatis* (n=3), *M. kansasii* (n=2) and *M. fortuitum* (n=1). All eight canine samples were found to be infected with *M. bovis.* Whilst the sensitivity of this assay for tuberculous infections was high (88.2%), future work is required to improve the sensitivity for diagnosis non-tuberculous mycobacterial infections.

2.1 Introduction

The identification of mycobacterial infections in human medicine has been dramatically improved by the introduction of molecular methods aimed to reduce the time to diagnosis as compared with culture (Dorn-In, 2019; Wang, 2019). For companion animals in the UK, histopathological examination of clinical samples to detect acid-fast bacilli (AFB) is almost always the first microbiological test for the diagnosis of mycobacterial infection (O'Halloran and Gunn-Moore, 2017). However, this method has a limited sensitivity and specificity and does not provide any information on the mycobacterial species (O'Halloran *et al.* 2016). In particular, microscopy cannot distinguish mycobacteria of the *Mycobacterium (M.) tuberculosis*-complex (MTBC) from the non-tuberculous mycobacteria (NTM) which have different zoonotic risks and optimal treatment protocols (Gunn-Moore, 2014).

Currently, specialist mycobacterial culture represents the gold standard and official reference method for the microbiological diagnosis of mycobacterial infections in companion animals (Middlemiss and Clark, 2018). Culture provides a pure isolate for subsequent genotypic identification and in some laboratories, antimicrobial susceptibility testing (Harvell *et al.* 2000). However, culture is affected by the growth rate of mycobacteria, especially for the slow-growing species which includes all of the MTBC organisms, as they need more than seven days to form a colony as compared with ~48–72 hours for the more rapidly growing mycobacteria (when starting with a pure inoculum) (Harvell *et al.* 2000). The slow growing species such as *M. bovis* and *M. microti* can take two to four months to isolate from tissue samples (Gunn-Moore *et al.* 2011a). In addition, infections due to some mycobacteria may not be detected by conventional culture because of the requirement of specific nutrients such as Mycobactin J for *M. avium* subspecies *paratuberculosis* (MAP), specific temperature culture conditions e.g. 30 °C for *M. marinum* or 42 °C for *M. xenopi*, whilst some mycobacteria, such as *M. lepraemurium,* remain uncultivable on synthetic media *in vitro* (Malik *et al.* 2013; Nakanaga *et al,* 2013).

Molecular methods have the potential to shorten the diagnosis from a minimum of several weeks to just days as well as offering the opportunity to widen the breadth of identifiable

species (Wang *et al.* 2019). The only molecular diagnostic test available to companion animals in the UK is available via the National Mycobacterial Reference Laboratories, e.g. Leeds University Mycobacterial Reference Laboratory (LUMRL), established and run to diagnose human cases of mycobacterial disease (O'Halloran *et al.* 2019). These laboratories have the advantage of using well-established methods for polymerase chain reaction (PCR) based diagnosis but lack validation for animal samples (O'Halloran *et al.* 2019). Due to the pathology of human tuberculosis as a predominantly pulmonary disease, human laboratory testing is optimised for testing small volumes of fresh sputum samples (e.g. GenoType MTBC; Hain Life-Sciences, Germany). The effect of formalin fixation and the use of whole tissue as the starting material on test performance (e.g. sensitivity and specificity) has not been assessed. A further drawback is the high financial cost of the test which has generally precluded its mainstream use for companion animals to date.

Culture data for UK cats has identified *M. microti* as the most frequently diagnosed mycobacterial infection, with *M. bovis* the second most common; therefore one third (34%) of UK feline mycobacterial infections are defined as tuberculous *i.e.* caused by organisms within the MTBC (Gunn-Moore *et al.* 2011a).

The consistent genetic differences between members of the MTBC are genomic regions of difference (RD) which can be used as markers to distinguish species within the complex (Teo *et al.* 2013). Though rare in companion animals, disease due to *M. tuberculosis* infection is extremely important to diagnose due to the inherent risk to human health. *M. tuberculosis* encodes eight RD, numbered 4-11, which have been lost from *M. bovis* through reductive evolution (Liébana *et al.* 1996; Huard *et al.* 2006). The MTBC organisms frequently isolated from feline tissue; *M. bovis* and *M. microti,* can be discriminated from one another by RD-1 (which encodes a number of virulence factors) and its loss is the reason for the relative attenuation of *M. microti* in humans (Pym *et al.* 2002).

Other than the MTBC, members of the *M. avium-intracellulare*-complex (MAC) represented the most frequently identified group of mycobacteria cultured from UK cats (Gunn-Moore *et al.* 2011a). The molecular identification of these organisms is more challenging than speciation

of the MTBC as it typically depends on the presence or absence of a variety of insertion sequences (IS) (Collins *et al.* 1997). The ability of these IS to move position within the genome sometimes generates mutations and so brings into question the sensitivity of individual IS sequences to identify particular MAC species (Kim *et al.* 2018). There are similar concerns about the specificity of these targets; for example, there has been a considerable debate surrounding the specificity of IS-900 to MAP (Englund *et al.*, 2002; Keller *et al.* 2002; Sohal *et al.* 2002; Johansen *et al.* 2005; Semret *et al.* 2006)

The principal aim of this study was to evaluate the usefulness of a novel mycobacterial gene PCR panel for use on companion animal formalin-fixed paraffin-embedded (FFPE) tissue biopsies. The gene panel was designed to include pan-mycobacterial markers; the heat shock protein 65kDA (*hsp65*) and 16S rRNA encoding genes along with the additional PCR targets of; the antigen 85 complex protein B (Ag85-B), RD-4, RD-1 and a reportedly MAC-specific IS, IS-1311.

2.2 Materials and Methods

This study was conducted with prospective approval from the School of Veterinary Medicine Ethical Review Committee at the University of Edinburgh and all relevant guidelines and regulations were adhered to throughout.

2.2.1 Tissue Acquisition

A total of 380 FFPE tissue biopsies of mycobacteria-induced granulomas were obtained from two groups of domestic cats.

The first group comprised 154 biopsies from cases diagnosed from across the UK that were treated for mycobacterial infections confirmed by at least one of; histological tissue examination, specialist mycobacterial culture, interferon gamma release assay (IGRA) and/or mycobacterial PCR performed by LUMRL. These cats were diagnosed and clinically managed under the supervision of the author and/or Professor Gunn-Moore with biopsy material

donated for the purposes of this research with informed owner consent once the material was no longer required for diagnostic and/or clinical purposes.

The second group of 226 samples came from cats that had tissue biopsies evaluated histologically between 2009 and 2015 at a large private diagnostic veterinary laboratory; Finn Pathologists, Norfolk. These samples were diagnosed by routine haematoxylin and eosin staining as containing pyo/granulomatous inflammation. Where possible, the aetiology was confirmed as mycobacterial by the identification of intra-lesional AFB with typical mycobacterial morphology.

Canine mycobacterial infections are diagnosed much less frequently in the UK compared to feline infections (Snider and Cohen, 1972). Therefore, canine biopsy material was collected on an *ad hoc* basis from different diagnostic laboratories and submitted by general practice veterinarians who could obtain owner consent for the use of the remnant tissue for this project. In total only eight canine tissue samples were analysed.

2.2.2 Selection of Genes and Primer Design

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is frequently used to normalise quantitative gene expression in RNA analyses as a so-called “housekeeping gene” due to its ubiquitous expression in mammalian cells (Leutenegger *et al.* 1999; Kipar *et al.* 2001; Taglinger *et al.* 2008). In this study, oligonucleotide primers (Table 2.1) specific for a section of the feline (297bp) and canine (230bp) GAPDH genes were designed to act as a control for successful extraction of amplifiable DNA from the FFPE biopsy samples. Primers specific for GAPDH genes were designed using the annotated nucleotide sequences of the reference genomes for the domestic cat (*Felis silvestris catus*; Ensembl accession number NM_001009307.1) and dog (*Canis lupus familiaris*; Ensembl accession number AF327898.1) respectively. Primers were assessed using genomic DNA extracted from remnant whole blood samples using the Qiagen DNeasy Blood and Tissue kit (Qiagen, Germany) according to the manufacturer’s instructions and the PCR amplification protocol as outlined below.

Six genes were selected for inclusion in a panel of mycobacterial genes. Firstly; a frequently analysed gene that can be sequenced for the speciation of mycobacteria, a 439 base pair (bp) partial section of the hsp65, which is present in all mycobacteria. It was amplified using primers Tb11 and Tb12 as first described by Telenti *et al.* (1993), shown in Table 2.1.

Table 2.1: The oligonucleotide primer sequences used in this study. hsp65: 65kDa heat shock protein as described by Telenti *et al.* (1993), Ag85-B: Antigen 85 complex protein B, ESAT-6: 6kDA early-secreted antigenic target, GAPDH: glyceraldehyde 3-phosphate dehydrogenase, bp: base pair.

Gene	Forward oligonucleotide sequence (5'-3')	Reverse oligonucleotide sequence (5'-3')	Expected product length (bp)
hsp65	ACCAACGATGGTGTGTCCAT	CTTGTCGAACCGCATACCCT	439
16s rRNA	ACTGAGATACGGCCCAGACT	TCACGAACAACGCGACAAAC	279
Ag85-B	TACCAGTCGGGACTGTCGAT	ACATGTCTGCGGCCTTGTAA	355
Rv1506c	TCTTGGAGTACCTGCGCTTG	ACTACGTTTCGAAGGTCGGG	290
ESAT-6	CCAGGGAAATGTCACGTCCA	ATGCGAACATCCCAGTGACG	233
IS-*1311*	CCCCTCGAACGACTCAACAA	AACGGATTGGTCGGCTGAAT	201
Feline GAPDH	CACCATCTTCCAGGAGCGAG	CTCCATGGTGGTGAAGACCC	297
Canine GAPDH	GTGAACGGGAAGCTCACTGG	TCCGATGCCTGCTTCACTAC	230

The second mycobacterial gene targeted alongside *hsp65* was the 16s rRNA gene, also common to all mycobacteria (Gürtler, *et al.* 2006). This was designed to amplify a smaller product than the hsp65 sequence in order to try to increase the sensitivity of the PCR assay.

The proteins A, B and C of the antigen 85 complex (Ag85) are among of those most abundantly produced by MTBC organisms in culture. Each protein is encoded by a separate gene within the genome and each has almost perfect sequence homology across the MTBC group Karbalaei-Zadeh-Babaki *et al.* 2017).

The Rv1506c gene encoded on the RD-4 genome segment is present in *M. tuberculosis* but has been lost from the other MTBC of importance in companion animals (*M. bovis* and *M. microti*), and so it was included as a screening marker to identify any potential *M. tuberculosis* cases (Ru *et al.* 2017). In order to discriminate *M. tuberculosis* and *M. bovis* from *M. microti*,

primers targeting the virulence factor 6kDa early-secreted antigenic target (ESAT-6) encoded on the RD-1 segment of the genome was included into the gene panel (Pym *et al.* 2002).

Finally, IS-1311 which is reportedly common to all of the MAC organisms, since it is present at high copy numbers per mycobacteria it is a likely sensitive diagnostic marker for this group of mycobacteria (Keller et al. 2002).

Apart from the *hsp65* gene, all other oligonucleotide primers were designed specifically for inclusion in this study using Primer3 software. Primers to identify pan-mycobacterial genes and those common to the MTBC were designed using the National Center for Biotechnology Information (NCBI) type strain genomes; *M. tuberculosis* H37Rv or *M. bovis* AF2122/97 as the reference sequence, whilst the NCBI type strain *M. avium* subspecies *paratuberculosis* K10 genome was used as the reference for IS-1311. As only short DNA fragments are usually recoverable from FFPE tissues all mycobacterial gene primers were designed to be 20bp long with a melting temperature of 59ºC±1ºC whilst generating a product of 300bp±150bp in size.

2.2.3 DNA Extraction from FFPE Tissues

Each FFPE tissue block to be tested was mounted onto a HM325 rotary microtome (Thermo Fisher Scientific, USA), and blocks were trimmed in to remove excess paraffin with a disposable Feather S35 microtome blade. Once the full tissue surface was exposed, three sequential 20µm sections (or all of the tissue if less than this was present) were cut and placed into a certified DNAse free 1.5mL screw-top Eppendorf tube. The paraffin was removed by incubating the cut sections in 1mL of xylene at 65ºC on a heat block for 15 minutes, the xylene was discarded and this step was then repeated. The tissue was rehydrated by resuspension through a sequential alcohol gradient comprising 100% ethanol, 99% ethanol, 95% ethanol and then 70% ethanol solutions. At each stage of the gradient, tissue was vortexed for 30 seconds and then incubated at room temperature for at least one hour. Between each step, samples were briefly centrifuged (10 seconds) at 6,000 x *g* and the supernatant was removed and discarded.

Rehydrated tissue was re-suspended in 300µL of an enzymatic digestion buffer (50mM Tris-HCl [pH7.5], 10mM EDTA, 0.5% sodium dodecyl sulphate [SDS] and 50nM NaCl) containing lysozyme (Sigma Aldrich, UK) to a final concentration of 20mg/mL which was incubated at 37ºC for a minimum of 48 hours. Proteinase K (Thermo Fisher Scientific, USA) was added to each of the samples to a final concentration of 300mg/mL, which were then incubated at 56ºC for one hour. The proteinase K was inactivated by heating the samples to 95ºC for 10 minutes. Macerated tissue was transferred, with the digestion buffer, to a 2mL tube pre-filled with sterile (acid washed) 0.1mm silica beads (Scientific Laboratory Supplies, UK). The samples were homogenised using a FastPrep bead beater (MP Biomedical, UK) setting P60 for 40 seconds following the addition of 180µL of tissue lysis buffer (Buffer ATL, DNeasy Blood and Tissue Kit; Qiagen, Germany).

Once samples had been returned to room temperature by incubation on ice, 200µL each of 100% ethanol and buffer AL (DNeasy Blood and Tissue Kit; Qiagen, Germany) were added and samples were mixed by vortexing for 20 seconds.

The tubes were centrifuged at 6,000 x *g* for one minute and the supernatant was transferred to a DNA isolation column (DNeasy Blood and Tissue Kit; Qiagen, Germany). The column was centrifuged at 6,000 x *g* for one minute and the collection tube containing flow through was discarded; 500µL of buffer AW1 (DNeasy Blood and Tissue Kit; Qiagen, Germany) was pipetted onto the column which was then centrifuged at 6,000 x *g* for one minute followed by the addition of 500µL of buffer AW2 (DNeasy Blood and Tissue Kit; Qiagen, Germany).

Columns were centrifuged at 8,000 x *g* for two minutes and then transferred to a clean 1.5mL DNase free Eppendorf tube. DNA was eluted from the column by adding 50µL of elution buffer (Buffer AE, DNeasy Blood and Tissue Kit; Qiagen, Germany) directly to the membrane and incubating at room temperature for five minutes. Elution of the DNA was performed by centrifuging the columns at 8,000 x *g* for one minute. DNA was quantified using a Nanodrop spectrophotometer ND-1000 and stored at -20ºC until needed for further analysis.

In addition to the mycobacteria containing tissue samples, a non-mycobacteria infected (*i.e.* negative) control skin sample was included. This tissue was a FFPE skin biopsy from a cat with a known cowpox virus infection that was mycobacteria-free.

2.2.4 PCR Amplification

All PCR reactions were performed in 96 well PCR plates in a final volume of 20µL containing; 10µL of GoldStar 2x PCR Mix (Eurogentec, Belgium), 0.15µL 25mM $MgCl_2$, 0.25µL 25pmol/µL combined forward and reverse oligonucleotide primers, 6.25µL nuclease-free water and 2µL of template DNA. Each PCR plate contained a positive template control for the gene being assessed (DNA extracted from reference strain culture colonies, described in Section 2.2.5), a sample of the DNA extracted from the non-mycobacteria skin sample as well as a no template (water only) negative control.

The PCR plates were sealed with adhesive plate sealant, centrifuged and transferred to a thermocycler. The same programme was used for all reactions comprising an initial step of heating the samples to 95°C for three minutes followed by 45 cycles of; 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds, followed by a final elongation step of 72°C for five minutes.

The final reaction samples were mixed with 2µL of 10x gel loading buffer (Thermo Fisher Scientific, USA) and analysed by 2% agarose gel electrophoresis containing SYBR Safe (Thermo Fisher Scientific, USA) at 120V for 45 minutes in order to visualise the products using GeneSNAP software and partner gel imager.

2.2.5 Culture and DNA Extraction from Mycobacterial Reference Strains

Reference strains of *M. bovis* (strain AF2122/97; ATCC BAA-935 obtained from Professor Jayne Hope, The Roslin Institute), *M. bovis* bacille Calmette–Guérin (*M. bovis*-BCG, strain 1173P2 [Pasteur], obtained from Dr Kirsty Jensen, The Roslin Institute) MAP (strain K10; ATCC BAA-968; obtained from Dr Heather Baird; The Roslin Institute) were grown in the containment level two and three (for *M.* bovis) laboratory facilities of The Roslin Institute,

University of Edinburgh. Single aliquots containing 1mL of mycobacteria suspended in culture media were added to 9mL of Middlebrook 7H9 media (Sigma Aldrich, UK) supplemented with Middlebrook ADC growth supplement (Sigma Aldrich, UK) to 10% by volume and 0.5g/500mL Tween 80 (Sigma Aldrich, UK). The culture media for MAP was additionally supplemented with 2 mg/L of Mycobactin J (Allied Monitor, Inc., USA).

Cultures were incubated at 37ºC/5% CO_2 with agitation in a shaking incubator for 14 days, after this time the optical density (OD) was measured daily using a spectrophotometer until an OD_{600} of 0.6 was reached, indicating that the mycobacteria had reached the exponential growth phase. At this time, the 10mL culture was added to a further 90mL of Middlebrook 7H9 ADC media and returned to the incubator. Again, the cultures were grown 37ºC/5% CO_2 with agitation for 14 days before daily assessment of the OD value. Once an OD_{600} of 0.6 had been reached, the mycobacteria were aliquoted into sterile 1mL screw-topped Eppendorf tubes and stored at -80 ºC until further use.

A single inoculum of *M. smegmatis* type strain (NCTC 8159 [Cornell 3], ATCC 19420; obtained from Dr Jo Stevens, The Roslin Institute) was grown overnight (16 hours) in 250mL of Middlebrook 7H9 media (Sigma Aldrich, UK) supplemented with Middlebrook ADC growth supplement (Sigma Aldrich, UK) to 10% by volume and 0.5g/500mL Tween 80 (Sigma Aldrich, UK) at 37°C/5% CO_2 with agitation. The following day it was confirmed that an OD_{600} of 0.6 had been reached and the mycobacteria were aliquoted into sterile 1mL screw-topped Eppendorf tubes and stored at -80 ºC until further use.

Genomic DNA was extracted from each species of mycobacteria as needed by thawing single 1mL aliquots and centrifuging the Eppendorf at 16,000 x *g* for 15 minutes to pellet the mycobacteria and the supernatants were discarded. The cells were resuspended in 180 µL of Enzymatic Lysis Buffer, comprising 20 mM Tris-Cl pH8.0, 2 mM sodium EDTA, 1.2% Triton X-100 and 20mg/mL lysozyme, prepared immediately before use). The solution was incubated at 37°C for at least 60 minutes before 25µL proteinase K and 200µL buffer AL (DNeasy Blood and Tissue kit; Qiagen, Germany) and mixed thoroughly by vortexing. Following an incubation at 56°C for 30 minutes; 200µL 100% ethanol was added and again mixed by vortexing. The

mycobacterial lysate was transferred to a Qiagen DNeasy column which was then centrifuged at 6,000 x *g* for 60 seconds. The flow-through was discarded from the collection tube, 500µL of buffer AW1 (Qiagen DNeasy Blood and Tissue kit) was added to the column which was then centrifuged at 6,000 x *g* for 60 seconds. The flow-through was discarded and this wash process was repeated with 500µL of buffer AW2 with centrifugation at 16,000 x *g* for two minutes. The column was dried by further centrifugation at 16,000 x *g* for two minutes. To elute the DNA from the column 50µL of buffer AE (Qiagen DNeasy Blood and Tissue kit) directly onto the membrane and left to incubate at room temperature for five minutes. The column was then placed into a nuclease-free Eppendorf and centrifuged at 6,000 x *g* for 60 seconds. The resulting DNA was analysed using a Nanodrop spectrophotometer ND-1000 and stored at -20°C when not in use.

The Roslin Institute does not currently hold Health and Safety Executive approval to culture *M. tuberculosis* so extracted DNA (from strain H37Rv; ATCC 25618) was obtained directly from Dr Robin Skuce, Agri-food & Biosciences Institute.

2.2.6 Sequencing and Molecular Speciation

All of the PCR primers used in this study were validated by sequencing of the product produced, similarly, where a PCR product was obtained for the mycobacterial hsp65 gene from a sample, this was retrieved for sequencing. The agarose gel containing the sample was visualised by ultraviolet trans-illumination and the product band was removed using a sterile disposable scalpel blade. The DNA was removed from the agarose using a PureLink Quick Gel Extraction Kit (Invitrogen, USA) by weighing the gel extract and adding three times the volume of gel solubilisation buffer (Buffer L3, PureLink Quick Gel Extraction Kit) and heating to 50^{o}C until fully dissolved.

One volume of isopropanol was added to the agarose solution and the solution was mixed by vortexing. The entire volume was then transferred to a DNA mini-column. Columns were centrifuged at 6,000*g* for a minute, the flow-through was discarded and 500µL of wash buffer (Buffer W1, PureLink Quick Gel Extraction Kit) was added to the membrane. Columns were

centrifuged at 6,000 x *g* for two minutes and the flow-through was discarded. The DNA was eluted into 50µL of nuclease free water by centrifugation at 8,000 x *g* for two minutes after incubating the membrane for five minutes at room temperature.

The quantity of DNA was estimated by comparing the original PCR band density to the known concentrations in the nucleotide ladder (1Kb DNA Ladder; Promega, USA) using ImageJ software. Purified DNA was also assessed using a Nanodrop spectrophotometer ND-1000 and diluted to a final concentration of 20ng in 30µL prior to being sent to the University of Dundee for Sanger sequencing by independent reactions using 3.2pmole of the forward oligonucleotide primer sequence (University of Dundee, 2019).

Generated sequence data was analysed using FinchTV software to obtain a consensus sequence. The mycobacterial species was determined by Basic Local Alignment Search Tool (BLAST) analysis.

2.2.7 Bacillary Grading System

The Ziehl–Neelsen (ZN) staining method was used to grade the number of AFB present in each biopsy. For the biopsy material examined by a private diagnostic pathology service, the pathologists' assessment was converted into a score (Table 2.2). For the blocks included via the Edinburgh cohort, tissue slides were produced, stained and analysed. For these tissues, 4µm thick sections were cut from the FFPE tissue samples using a HM325 Rotary Microtome with disposable Feather S35 blades (Thermo Fisher Scientific, USA). These were mounted onto Superfrost Plus slides (Thermo Fisher Scientific, USA) and incubated in an oven at 65°C for a minimum of 16 hours. The tissue sections were dewaxed and rehydrated, flooded with filtered dilute carbol-fuschin stain containing phenol (ProLab Diagnostics Inc., UK) and heated over a flame inside a vented fume-cabinet until visible fumes were produced. The slides were left to stain for ten minutes. Following thorough rinsing with water, decolourisation was carried out using an acid-alcohol solution of 5% sulphuric acid by volume diluted in methanol. The slides were counterstained by immersing in haematoxylin for seven seconds, followed by Scott's Tap Water for seven seconds. The tissue sections were dehydrated and coverslips

were mounted using Pertex mounting medium (Pioneer Research Chemicals Ltd., UK). Slides were examined under oil emersion light microscopy and the number of AFB was graded 0-5 using a modified scoring system, similar to those previously reported for bovine and companion animal samples (Malik *et al.* 2002; Garbaccio *et al.* 2019), detailed in Table 2.2.

Table 2.2: Grade descriptors for the modified mycobacterial burden grading system used in this study. Grades were based on the evaluation of three HPF fields. *HPF; high power field (oil emersion, light microscopy), AFB; acid fast bacilli.*

Score	Finn Pathologist description of mycobacterial burden	Approximate number of mycobacteria
0	Negative	None seen in section examined
1	Rare/solitary	Single AFB per HPF or fewer
2	Few/scattered	Up to ten AFB per HPF
3	Moderate	Ten to 50 AFB per HPF
4	Large number	50-100 AFB per HPF
5	Vast/Innumerable	Too many AFB to count accurately
6	Not counted	Insufficient material after PCR to perform staining protocol

2.3 Results

2.3.1 PCR Assay

A cohort of 380 feline and eight canine FFPE biopsy samples were examined using the novel mycobacterial PCR protocol developed in this study. Of the feline samples examined, 154 cats had been treated under the supervision of the author and/or Professor Gunn-Moore. All of these cases were referred due to the clinical suspicion of mycobacterial infection based on the presence of compatible clinical signs that included combinations of; single or multiple skin nodules/masses, sometimes with enlarged local lymph nodes, unexplained weight loss and/or respiratory disease with typical pathology visible on diagnostic imaging. Pulmonary interstitial disease was the most frequently reported abnormality diagnosed on thoracic imaging although bronchial changes were seen occasionally; only rarely were pulmonary tubercles visible.

Mycobacterial infection was confirmed in the University of Edinburgh sub-set by identifying AFB with mycobacterial morphology on histological examination of diseased tissue alone in only 36 cats (of 154, 23%). The remaining cats had at least one diagnostic test performed (independent to this study), these included an interferon gamma release assay (IGRA; n=71, 46%), mycobacterial culture (n=49, 32%) and/or PCR at LUMRL (n=8, 5%). Ten cats had both an IGRA and culture performed, but all were culture negative so the IGRA diagnosis was accepted. Four cats had an IGRA and PCR test performed which were in agreement, and no cats had both PCR and culture performed. Based on the results of these independent mycobacterial test results, 118 cats of these 154 were given a specific diagnosis comprising 60 *M. microti*, 36 *M. bovis,* 14 MTBC infections (without further discrimination), four MAC and one each of *M. fortuitum, M. kutatamoesnse, M. malmoense* and *M. chelonae* infections.

Tissue blocks collected from Finn Pathologists, Norfolk all had a histological finding of (pyo)granulomatous inflammation suggestive of mycobacterial infections. The overwhelming majority (219, 97%) of these lesions were associated with AFB that displayed typical mycobacterial morphology. No specific aetiological agent was observed in the remaining seven tissue samples when sections were stained with ZN, Periodic acid–Schiff and Giemsa

stains. However, the granulomatous inflammatory infiltrate in all seven was dominated by aggregates of reactive macrophages surrounded or interspersed with moderate numbers of neutrophils and surrounded by small numbers of lymphocytes, so any cause other than mycobacterial infection was deemed to be extremely unlikely and therefore all samples in this study were included as mycobacteria-infected.

Histopathological examination identified AFB with mycobacterial morphology in all of the canine samples. All eight of the dogs were infected with *M. bovis,* as indicated by IGRA and confirmed by specialist mycobacterial culture in two cases.

Primers targeting the feline and canine GAPDH gene were tested using feline and canine genomic DNA respectively, extracted from remnant whole blood samples. Both of the primer sets selected produced only a single amplicon of the predicted product size and resulted in no non-specific amplification under no template control conditions, Figure 2.1.

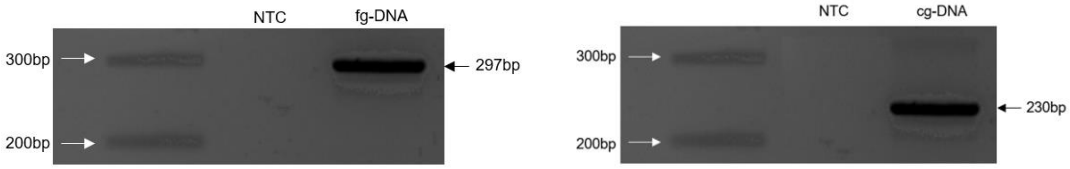

Figure 2.1: The GAPDH gene product was successfully amplified from feline (297 base pairs [bp], left) and canine (230bp, right) genomic DNA (fg-DNA and cg-DNA respectively) templates and not from a no template control (NTC). PCR products (black arrows) were loaded into a 2% agarose gel, alongside a 1Kb DNA ladder (200bp and 300bp markers are indicated by white arrows).

Once DNA had been extracted from all 380 feline and eight canine tissue biopsies, all samples were shown to contain DNA by Nanodrop spectrophotometer ND-1000 analysis. To establish that the DNA was amplifiable by PCR, amplification of GAPDH from host genomic DNA was attempted. This was successful from 346 (91%) of the feline samples and all eight (100%) canine tissues.

The set of six mycobacterial genes were chosen for inclusion in the diagnostic mycobacterial panel; *hsp65*, 16s rRNA, Ag85-B, Rv1506c, ESAT-6 and IS-1311. A total of 30 primer pairs were screened against DNA from the reference isolate mycobacteria; *M. tuberculosis* H37Rv, *M. bovis* AF2122/97, *M. bovis*-BCG Pasteur (the present tuberculosis vaccine strain), MAP

type strain K10 and *M. smegmatis* type strain ATCC 607. The final panel of primers (Table 2.1) were selected as those which produced only a single PCR product which matched the predicted size (determined by agarose gel electrophoresis, Figure 2.2), with no amplification under no template control conditions or from the feline tissue sample not infected with mycobacteria. In addition, when the amplified product was extracted and sequenced, it was required to match the reference sequence as determined by BLAST analysis.

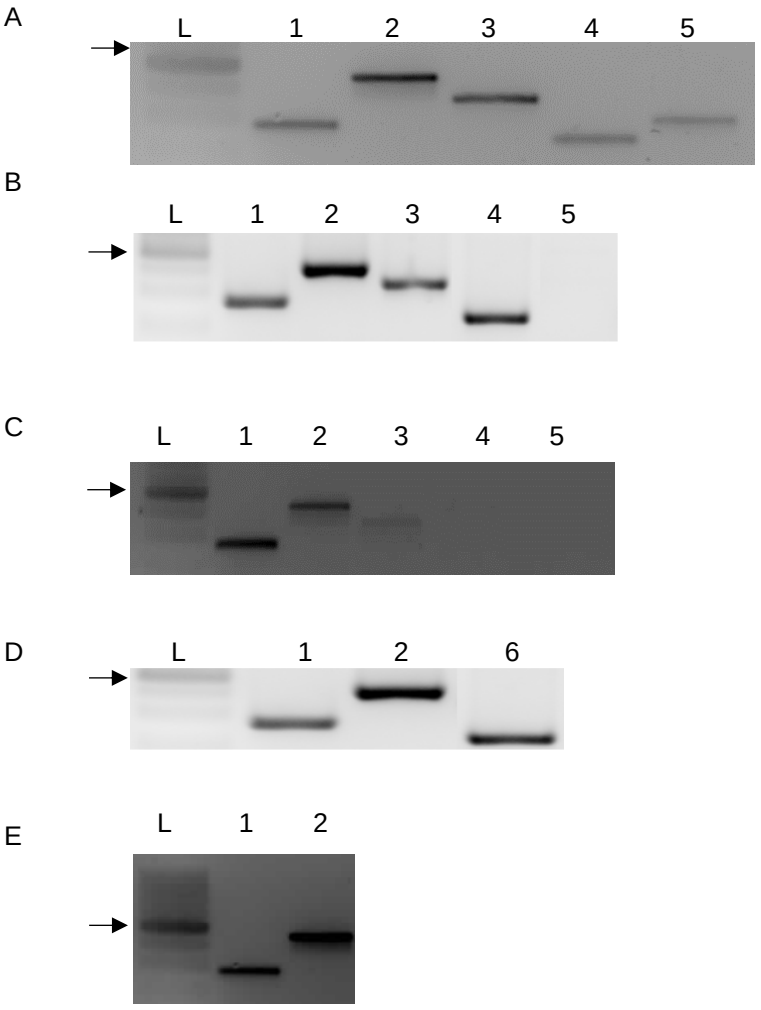

Figure 2.2: Products produced by the final six-gene mycobacterial panel primers selected analysed by 2% agarose gel electrophoresis alongside a 1Kb DNA ladder (L, black arrows indicate 500bp marker). The anticipated pattern of bands was found to be present in the DNA extracted from *M. tuberculosis* H37Rv (A), *M. bovis* AF2122/97 (B), *M.* bovis-BCG Pasteur (C) MAP strain K10 (D) and *M. smegmatis* type strain ATCC 607 (C); 16s rRNA (lane 1, 279bp), *hsp65* (lane 2, 439bp), Ag85-B (lane 3, 355bp), ESAT-6 (lane 4, 233bp), Rv1506c (lane 5, 290bp) and IS-1311 (lane 6, 201bp).

A total of 346 feline samples where amplifiable DNA was extracted were analysed using the six-gene mycobacterial panel and produced gene products (Figure 2.3); 59 (17.1%) produced an *hsp65* gene product whilst 270 (78.0%) were positive for 16s rRNA. All samples giving rise to an *hsp65* product also produced a 16s rRNA amplicon.

Of the 346 samples, 145 (41.9%) were positive for Ag85-B, diagnosing tuberculosis, of which no (0%) samples gave an Rv1506c product (*i.e.* there were no *M. tuberculosis* infections) whilst 39 (11.3% of 346) samples were also positive for ESAT-6, consistent with a diagnosis of *M. bovis*. These results indicate that 106 samples (30.6%) were infected with *M. microti* (*i.e.* they were Ag85-B positive but ESAT-6 negative). All of the samples from which Ag85-B and ESAT-6 products were amplified also produced 16s rRNA amplicons. Only two (0.9%) cat biopsies amplified DNA for IS-1311 (consistent with MAC infections)*;* both also produced 16s rRNA products, but only one amplified *hsp65.*

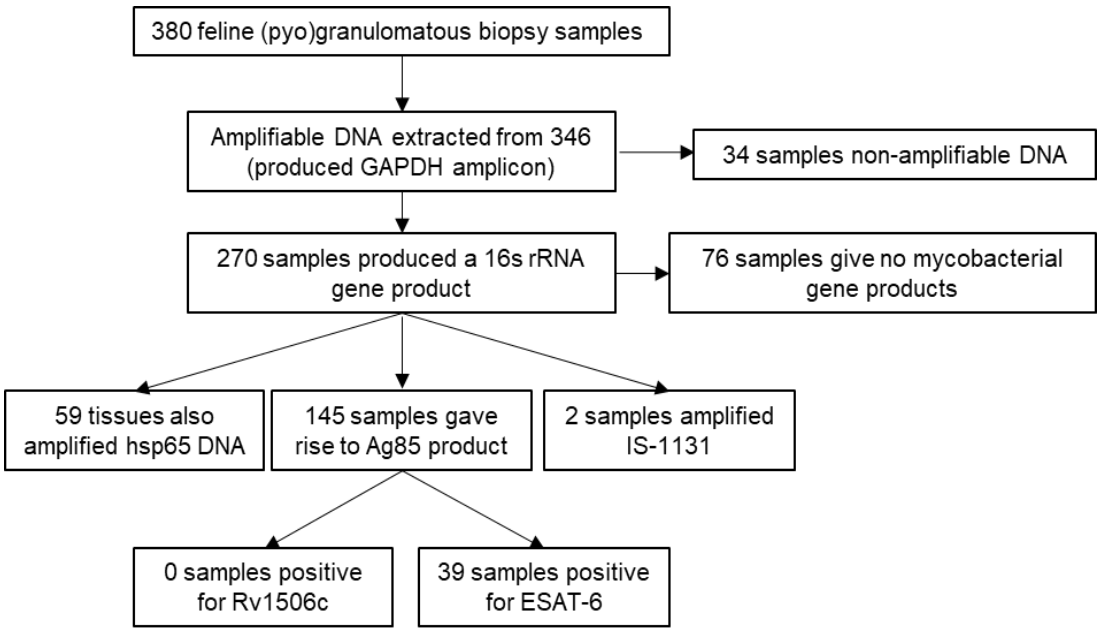

Figure 2.3: A flow diagram demonstrating the results generated from sequential PCR amplification of feline GAPDH and the six-gene mycobacterial panel for the feline biopsy samples used in this study.

In total, 59 of the feline samples analysed produced an amplicon for the *hsp65* gene fragment that has been used in previous studies to speciate mycobacterial infections (Telenti *et al.* 1993, Figure 2.4). The *hsp65* PCR products were extracted from the agarose gels, purified and sequenced using the forward primer sequence by the University of Dundee (Figure 2.5). The consensus nucleotide sequence generated was entered into a BLAST search to match it back to any registered genomes. Of the 59 *hsp65* sequences generated, 19 sequences most closely matched the sequences for *M. tuberculosis* H37Rv, Beijing 2014 and similar clinical isolates, *M. tuberculosis* variant *bovis* strain 1 (more commonly referred to as strain AF2122/97) and *M. bovis*-BCG, as the *hsp65* gene has a conserved sequence across the MTBC organisms (Borgers *et al.* 2019). An additional nine *hsp65* sequences matched to specific mycobacterial sequences in the NCBI database; three *M. malmoense;* three *M. smegmatis,* two *M. kansasii* and one *M. fortuitum.* For all 28 samples matched to a species, the nucleotide sequence identity was high, ranging from 85.71% to 96.22% (median 95.99%).

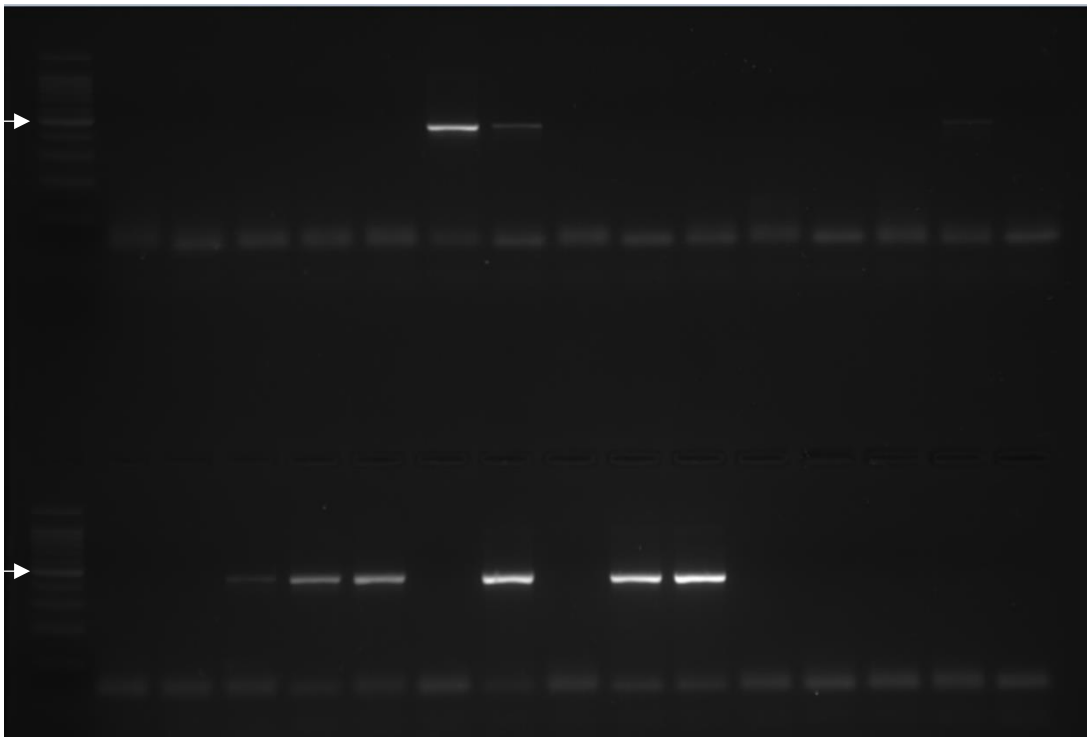

Figure 2.4: A representative image of *hsp65* gene products amplified from template DNA extracted from feline FFPE biopsy tissues, analysed by 2% agarose gel electrophoresis against a 1Kbp DNA ladder (500bp markers are indicated by white arrows). In this image, nine of the 30 samples show amplification of a gene product (439bp) with variable band intensity.

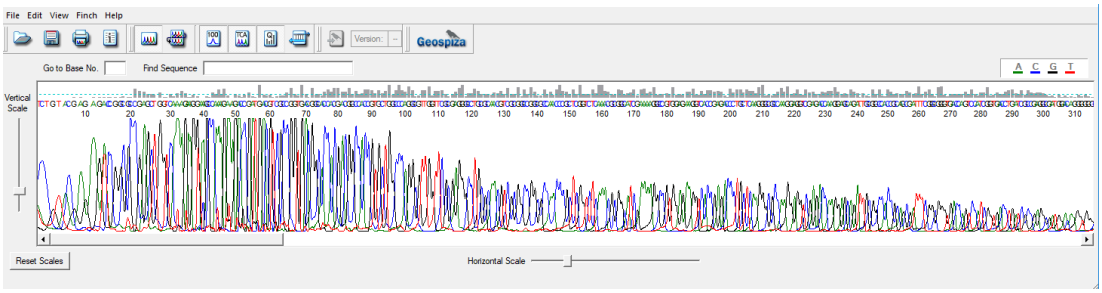

Figure 2.5: A representative *hsp65* gene sequencing trace displayed using FinchTV software. The trace was obtained from extracting and sequencing the *hsp65* gene product from agarose gel as shown in Figure 2.4. The consensus nucleotide sequence for the sequencing trace was extracted and matched to recorded sequences in the NCBI database by BLAST search.

Of the remaining hsp65 gene products, nine sequences could only be identified to the genus level as belonging to a *Mycobacterium* species, but the species was not identifiable, and for the final 22 samples, no significant similarity was found between the consensus nucleotide sequence and any genome within the NCBI database.

As described above, 119 cats with biopsy material available to this study had previously received a species diagnosis from independent mycobacterial testing; of the 60 diagnosed with *M. microti* infections, 53 (88.3%) were similarly diagnosed in this study. A group of 36 cats were previously diagnosed with *M. bovis* infections, which was matched in this study for 30 (83.3%) of them. For 14 cats, where previous diagnostic testing only identified their infections as belonging to the MTBC group, this study, based on the PCR results for ESAT-6, identified nine as *M. bovis* and five as *M. microti.* Therefore, this study identified MTBC infections to the species level in 97 cases of the 110 (88.2% sensitivity) biopsies that had previously been independently diagnosed as MTBC. The remaining 13 samples were in the group of biopsies which produced a 16s rRNA gene product, confirming mycobacteriosis, but no additional mycobacterial genes were identified.

Eight cats had been previously diagnosed with non-tuberculous mycobacterial infection to the species level. Four cats had been diagnosed with MAC infections of which two (50%) were found to be PCR positive for IS-1311 in this analysis. The individual cats with *M. fortuitum* and *M. malmoense* infection were both successfully identified as these species in this study by

hsp65 gene sequencing in this study. This meant that the protocol developed in the current had a 75% sensitivity for identifying non-tuberculous mycobacterial infections.

Of the eight canine samples from dogs previously diagnosed with *M. bovis* infections, just one produced an *hsp65* gene product (12.5%). When sequenced, this shared 99.98% sequence identity with *M. tuberculosis* variant *bovis* strain 1 (*i.e. M. bovis* AF2122/97). All eight samples (100%) produced 16s rRNA, Ag85-B and ESAT-6 amplicons but not Rv1506c or IS-1311.

2.3.2 Bacillary Grading

All tissue samples were assigned a mycobacterial burden grade based on i) evaluation by a single diagnostic laboratory (Finn Pathologists, Norfolk) or ii) the number of AFB visible on ZN stained slides examined by light microscopy under oil emersion. A five point system was developed for slides stained as part of this study (exemplified in Figure 2.6) from paucibacillary infections with no visible AFB (Grade 0) or sporadic individual bacilli (Grade 1) to multibacillary infections associated with very large numbers of AFB (Grade 5).

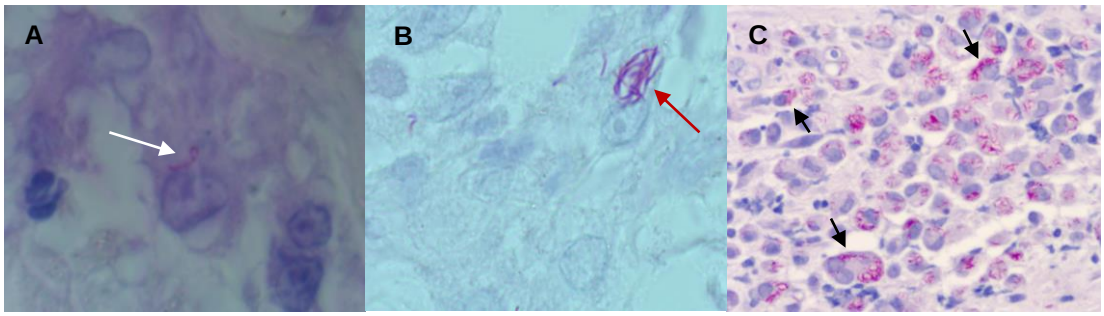

Figure 2.6: Images taken from representative sections of slides assigned different mycobacterial grades. Slide A was scored at Grade 1 as it only contains a single AFB (white arrow). Slide B was assigned Grade 3, a number of cells with macrophage morphology contain moderate numbers of AFB (red arrow). Slide C was assigned Grade 5 due to the large accumulations of AFB (black arrows) within multiple aggregates of macrophages throughout the section. All sections were stained following the ZN protocol. Slides were visualized under oil emersion x100 magnification for sections A and B or x40 magnification for section C and images were taken with Zen Blue software.

For 44 of 380 (11.6%) feline FFPE tissue samples, there was insufficient tissue remaining following PCR analysis to complete the ZN stain and to assign a mycobacterial grade. A small number (11, 2.9%) of tissue sections did not contain any visible AFB. Of the AFB containing

sections 44 (11.6%) were assigned Grade 1, 122 Grade 2 (31.9%), 62 Grade 3 (16.4%), 36 (9.5%) were given a Grade 4 and 61 (15.9%) were allocated Grade 5.

In order to test the hypothesis that higher grade samples would be more likely to give a positive PCR result, the percentage of samples within each grade from which a 16s rRNA PCR product (as the most frequently identified gene) was successfully obtained was compared using the Chi-squared (χ^2) statistical test. Statistical analysis showed significantly unequal distribution of positive results ($\chi^2 = 131$, $p<0.001$) with samples graded 0 and 1 significantly less likely to give a positive PCR product for 16s rRNA than samples assigned higher grades.

2.4 Discussion

The data presented in this study represents the first reported attempt to develop a PCR assay for the speciation of mycobacterial infections focussing on the UK companion animal population. Previously published PCR protocols developed for companion animal mycobacteriosis have primarily been used for diagnosing lepromatous diseases; in particular canine leproid granuloma (CLG) and feline leprosy syndrome (Foley *et al.* 2002; Huges, *et al.* 2002; Conceicao *et al.* 2011; Reppas *et al.* 2013; O'Brien *et al.* 2017). Both of these clinical entities are frequently diagnosed in countries without endemic tuberculosis in cattle or wildlife (and therefore cats), such as Australia, and so there has not been a need for assays which include MTBC targets (Malik *et al.* 2013; Reppas *et al.* 2013; O'Brien *et al.* 2017). By contrast, MTBC infections represent the most commonly identified feline mycobacterial infections in UK cats (Gunn-Moore *et al.* 2011a).

In each of the previously described protocols, the majority of DNA samples were extracted from fresh tissue, methanol-fixed stained or unstained cytology samples (typically aspirates of cutaneous nodules) with a minority being derived from FFPE tissue (; Foley *et al.* 2002; Huges, *et al.* 2002; Conceicao *et al.* 2011; Reppas *et al.* 2013; O'Brien *et al.* 2017). In the UK, samples suspected of originating from animals with mycobacterial disease are nearly always formalin fixed due to the concern that they may contain viable *M. bovis* or *M. microti* which are defined by the UK Health and Safety Executive as Hazard Group 3 organisms, which demarcates

pathogens that can cause severe human disease and can spread through the community easily (HSE, 2013). The fixation of tissue in formalin can lead to a reduction in the sensitivity of DNA based diagnostic techniques due to the damage and fragmentation of DNA which inevitably occurs, especially when the contact time between the tissue and fixative exceeds 48 hours (Malik *et al.* 2013). However, a molecular assay for the detection of Mycobacterium spp. DNA within CLG FFPE specimens was successful in all 24 samples tested (Reppas *et al.* 2013).

Nearly half of the samples used in this study were FFPE samples acquired retrospectively from a variety of diagnostic laboratories from across the UK and so tissue processing, and notably the duration of tissue fixation, was not a controllable factor. Despite this, GAPDH was amplifiable from 91.1% of feline samples and 100% of canine samples. Similarly, the sensitivity for MTBC genes within feline tissues was relatively high; 88.3% for *M. microti,* 83.3% for *M. bovis* and 88.2% for the MTBC overall, and 100% for canine samples, though the canine sample size was very small. Compared to the currently available feline IGRA, this PCR test has a lower sensitivity for MTBC which is reported as 100% for the IGRA but has comparable sensitivity for *M. bovis* which one study found to be 80% for the IGRA (Rhodes *et al.* 2008; Rhodes *et al.* 2011). Similar to the IGRA, the sensitivity of the assay developed in this study only identified 50% of the cats infected with MAC organisms (Rhodes *et al.* 2008; Rhodes *et al.* 2011). The reason for this is not immediately clear but it may be a result of choosing a single IS (IS-1311) as the target to identify this group; due to the documented problems of sensitivity and specificity of IS PCR targets for the MAC it may be necessary to include a combination of several such targets into the gene panel to improve the test accuracy (Englund *et al.*, 2002; Keller *et al.* 2002; Sohal *et al.* 2002; Johansen *et al.* 2005; Semret *et al.* 2006). Although the LUMRL PCR is used clinically for feline mycobacterial samples in the UK, its performance has not been systematically evaluated for companion animal tissues and so it is not currently possible to compare sensitivities with the PCR panel developed here.

Further work to enhance the sensitivity of this assay would be beneficial if this test were to be used clinically. As formalin fixation fragments DNA, targeting smaller regions of the genes of

interest may yield higher rates of positive results. The PCR products used in this assay are currently between ~200bp and ~450bp in size, which is relatively large. One possibility for future development would be to target internal regions of the current gene products by quantitative PCR (qPCR), as this typically amplifies much smaller segments of DNA (e.g. between 80bp and 150bp) and a similar qPCR approach has already been successful for the diagnosis of CLG. A potential drawback of a qPCR approach however would be the probable loss of sequencing ability. Whilst it would still be possible to purify and sequence any gene products, the smaller size would inevitably reduce the potential number of nucleotide variations present, so there would be a reduction in the ability of the product sequences to assign a species diagnosis. As is currently the case with the *hsp65* gene sequencing in this study, the MTBC genome homology means that sequencing these products is less useful than determining the presence, or absence, of specific RD, this reduction in gene product size could be limiting in terms of identifying the less commonly speciated mycobacteria *i.e.* those outside the MTBC and MAC.

However, *hsp65* gene sequencing was only possible from 15.5% of feline tissues and 12.5% of canine samples in this study. The sequence data obtained did not add diagnostic information in more than half of these (31/59) as the sequence was either not found in the NCBI database or was only identifiable to the genus level, which had already been demonstrated by the amplification of the product. Furthermore, for cases where *hsp65* gene sequencing identifies MTBC sequences (19 cats and all eight dogs in this study population), further molecular diagnostics are needed to discriminate between *M. tuberculosis, M. bovis* and *M. microti* as these organisms pose markedly different zoonotic risks to the owners of infected animals, so this further distinction is critical. The results of *hsp65* gene sequencing were most beneficial for the nine cases of non-tuberculosis mycobacteriosis caused by *M. malmoense, M. smegmatis, M. kansasii* and *M. fortuitum* that would not have otherwise been identified by this study. However, these nine cats only represent 2.4% of the total population of 380 samples. Consequently, it may be considered a worthwhile trade off to move to a qPCR based system with an overall increase in sensitivity but losing the ability to speciate some of the less commonly encountered mycobacteria in UK cats. It may also be possible to identify

gene targets that could be specific to these organisms and add them to any new diagnostic panel, especially as *M. malmoense* and *M. fortuitum* represent the most abundantly identified species from the mycobacterial culture of feline biopsies after the MTBC and MAC organisms.

Only one published study has examined the results of mycobacterial culture in relation to feline samples in the UK and none have examined canine submissions. In that publication, Gunn-Moore *et al.* (2011a) examined 339 culture results which is a comparable size to the findings presented here (n=380). The study reported that *M. microti* was isolated from 18.6% of submissions, *M. bovis* from 15.3% and MAC from 7.4% (including both *M. avium* and *M. intracellulare*). These organisms accounted for 41.3% of all submissions and 88% of culture-positive samples, whilst 53.1% of submissions yielded no growth (Gunn-Moore *et al.* 2011a). In contrast, the data presented in this study identified gene patterns consistent with *M. microti* in 30.6% of the tissues tested which is significantly higher than the results produced from tissue culture, meanwhile the frequency with which *M. bovis* infections were identified is similar between the two methods, 15.3% compared to 11.3% respectively.

One reason for the discrepancy in prevalence with respect to *M. microti* could be the relative difficulty in culturing this organism, which has remained problematic since the original studies by Wells from the late 1930's (Wells and Oxon, 1937; Wells, 1946). The very slow *in vitro* growth of *M. microti* compared to other organisms within the MTBC, and its variable tolerance to glycerol means that to be sensitive, culture methods require markedly extended incubation times for clinical samples, typically 12-16 weeks compared to the 4-8 weeks for *M. bovis*, plus the use multiple media types, both with and without glycerol (Wells and Oxon, 1937; Wells, 1946; Xavier *et al.* 2007). It is therefore likely that the previously reported prevalence of *M. microti* in UK cats based on culture results is an underestimate and it may in fact be significantly higher than previously thought. Although *M. microti* is relatively avirulent in humans, a number of people have been infected and of particular concern is that a number of these patients were immunocompetent (as far as their physicians could assess) which raises questions with regards to the potential for zoonotic transmission from cats (Pym *et al.*, 2002; Xavier *et al.* 2007). Despite these concerns, it remains the case that to date there are no instances of zoonotic transmission of *M. microti* from cats in the literature.

Importantly in this study, no *M. tuberculosis* infections were identified in any of the feline samples tested. This is likely to be because cats seem to be resistant to developing clinical disease due to *M. tuberculosis,* as has been demonstrated in experimental challenge studies, but whether or not they can carry latent infections remains unproven (Francis, 1958). Similarly, no dogs in this study were found to have *M. tuberculosis* infections. However, the canine sample size was very small and so may not be representative of all canine mycobacterial infections in the UK. Tuberculosis in dogs has historically been reported following both *M. tuberculosis* and *M. bovis* infections, with canine cases of *M. microti* occurring only very rarely (Snider and Cohen, 1972; Deforges *et al.* 2004). It is therefore an important benefit of this assay that the distinction between *M. tuberculosis* and *M. bovis* can be accurately made. For the first time, the MTBC organisms were identified in more than half of the feline samples; 54.0% of the 270 that were positive for at least one mycobacterial gene demonstrating that veterinary clinicians in the UK should have a high suspicion of tuberculosis in cases with compatible lesions.

Organisms of the MAC were rarely identified in this study, only 0.5% of the total 380 samples were positive for IS-1311, significantly lower than the percentage diagnosed by culture results. However, this probably reflects a lack of sensitivity of this marker rather than any significant decline in prevalence. For example, independent testing (IGRA and/or LUMR PCR) had already identified that twice the number of cases included in this study were infected with MAC organisms than this assay indicated. Further work is therefore needed to improve the sensitivity to detect MAC infections before any conclusions can be made regarding this result.

In agreement with previous studies considering feline mycobacterial diseases, the number of AFB in these sections was highly variable (Gunn-Moore *et al.* 2011b; Malik *et al.* 2013). Herein, the overwhelming majority (97%) of samples collected from the primary diagnostic lab (Finn pathologists) contained visible AFB which likely reflects the time given by these veterinary pathologists to search granulomatous sections for foci of AFB. This is a significant increase on a previous UK study in which primary diagnostic laboratories found AFB in 64% (125/195 cats) of the cases tested (Gunn-Moore *et al.* 2011b), suggesting pathologists may be more

aware of the significance of mycobacterial disease in UK cats and therefore they might be more likely to persist in examining suspect sections for AFB.

This study found that although AFB numbers were inconsistent between sections, when ZN slides were scored, by far the most frequently attributed grade was Grade 2, defined as those slides that contained few or scattered organisms and/or up to ten AFB per high power field under oil emersion light microscopy. This finding is consistent with the large prevalence of tuberculous infections in this study population that are typically paucibacillary. As was initially hypothesised, those sections which were graded as Grade 1 or 2 were significantly less likely to give a positive PCR result for the 16s rRNA gene than sections that scored a higher grade, probably because the amount of mycobacterial DNA in the template material was greater for these samples. Importantly however, it was possible to amplify genes from some samples in every group, including those assigned Grade 0 (negative for AFB). This has also been demonstrated for samples analysed by LUMRL and in terms of culture, 41% of samples that were AFB negative were successfully cultured (Gunn-Moore *et al.* 2011a; Sharp *et al.* 2019). This may reflect either the imperfect sensitivity of the ZN method for staining all mycobacteria present in a sample or it may be simply that the section stained and examined contained no organisms but that they existed elsewhere in the lesion, so scoring sequential sections may have given different results. In either case, it is clinically important that a molecular diagnosis can still be possible even with a negative ZN stain, particularly if the entire lesion has been fixed and so mycobacterial culture is impossible.

In conclusion, this study successfully developed a molecular method for the diagnosis of feline and canine mycobacterial infections in the UK. This six-gene mycobacterial assay identified a significantly higher proportion of cats with *M. microti* infections than previous studies, indicating that the prevalence of this infection may have been underestimated previously. Critically, no *M. tuberculosis* cases were identified and so the current UK wide advice that companion animal mycobacterial infections pose very low risk to their owners appears to be justified. This assay was found to have high sensitivity (88.2%) for the MTBC group of mycobacteria but was low (50%) for the MAC group, so future work to improve the sensitivity further is warranted.

2.5 References

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Chapter 3: Cytokine and Chemokine Concentrations as Biomarkers of Feline Mycobacteriosis.

Preface

The following Chapter presents the findings of a study, conducted as part of this studenship programme that has been published in the journal *Scientific Reports.*

The manuscript derived from this Chapter is available as an eponymous Open Access article: O'Halloran, C., McCulloch, L., Rentoul, L., Alexander, J., Hope, J.C. and Gunn-Moore D.A. (2018) Cytokine and Chemokine Concentrations as Biomarkers of Feline Mycobacteriosis. *Scientific Reports.* 8, Article number: 17314.

Author contributions:

C. O'Halloran, the candidate, conducted the experiment, performed the data analysis and wrote the manuscript. Dr McCulloch and Mr Rentoul provided technical assistance conducting the assays. Dr Alexander provided the study with control animal sera. Professors Hope and Gunn-Moore jointly supervised and secured funding for the study.

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Abstract

Mycobacteriosis is an emerging zoonotic disease of domestic cats and timely, accurate diagnosis is currently challenging. To identify differential cytokine/chemokine concentrations in serum/plasma of cats, which could be diagnostic biomarkers of infection we analysed plasma/serum from 116 mycobacteria-infected cats, 16 healthy controls and six cats hospitalised for unrelated reasons using the Milliplex MAP Feline Cytokine Magnetic Bead multiplex assay. Three cytokines; sFAS, IL-13 and IL-4 were reduced while seven; GM-CSF, IL-2, PDGF-BB, IL-8, KC, RANTES and TNF-α were elevated in mycobacteria-infected cats compared to healthy controls. However, IL-8 and KC concentrations were not significantly different from cats hospitalised for other reasons. Elevations in TNF-α and PDGF-BB could have diagnostic potential to identify *M. bovis* and *M. microti* infected cats specifically while GM-CSF, IL-2 and FLT3L were increased in MTBC infected cats. This study demonstrates potential use of feline tuberculosis as a spontaneously occurring model of this significant human disease. Cytokine profiling has clear diagnostic potential for mycobacteriosis of cats and could be used discriminate tuberculous from non-tuberculous disease to rapidly inform on zoonotic risk. Future work should focus on the in-field utility of these findings to establish diagnostic sensitivity and specificity of these markers.

3.1 Introduction

Tuberculosis has been the cause of considerable morbidity and mortality in the human population for centuries, and remains the leading cause of death from any single infectious agent (*Mycobacterium (M.) tuberculosis*) worldwide [1–3]. Historically, the significance of this disease has resulted in extensive biomedical research interest and, due to the growing threat of drug resistance, tuberculosis is a major focus of collaborative global healthcare investment [4–8]. Meanwhile, tuberculosis in domestic cats receives relatively little research attention, despite being more common and clinically significant than previously thought [9–11]. In the UK, approximately 1% of all feline biopsies submitted for routine histopathological analysis show changes consistent with mycobacteriosis and a third of these contain Ziehl-Neelsen (ZN) positive organisms when stained, with morphology indicative of the presence of mycobacteria [10,12].

The various mycobacterial species that have been identified in companion animals, including cats, can be grouped into the same two major categories as human mycobacterial disease; those belonging to the *Mycobacterium tuberculosis*-complex (MTBC) and the non-tuberculous mycobacteria (NTM, also referred to as 'atypical mycobacteria' or 'mycobacteria other than tuberculosis', MOTT) [11,13–19].

The MTBC consists of ten highly genetically related species of mycobacteria which are capable of causing tuberculosis in both man and other animals, and are some of the oldest recorded zoonotic diseases known to both human and veterinary medicine [20–26]. All member species of the complex share identical sequences across the 16s rRNA gene and 99.5% sequence homology across the remainder of the genome [27]. The most discriminating features between the species at the nucleotide level are genomic deletions, termed regions of difference (RD) [28–31]. The MTBC organisms which infect cats, *Mycobacterium microti* and *Mycobacterium bovis*, differ by RD-1 [12,32–34]. This region is absent from *M. microti* but present in all other MTBC organisms [28]. It has been shown to encode a variety of molecules which act as virulence factors which are also immuno-dominant proteins such as; 6kDa early-secreted antigenic target (ESAT-6) and the 10kDa culture filtrate protein (CFP-10) [35–37].

In companion animals, such as cats, MTBC infections pose a potential zoonotic risk to their owners; additionally these cases may act as a potential source of environmental contamination with mycobacteria such as *M. bovis*, a pathogen of major animal health significance in the UK and other countries as the causative agent of bovine tuberculosis (bTB) [38–41].

In stark contrast to human tuberculosis cases, the majority of feline tuberculosis cases present with localised nodular cutaneous disease, frequently with a degree of ulceration and occasionally with a draining sinus tract [9,13,14,34]. The lesions are typically distributed around the face, extremities and tail base – the so-called "fight and bite sites" [15,34]. Skin lesions may be accompanied by a localised or even generalised lymphadenopathy, or lymphadenopathy (usually of the submandibular, pre-scapular or popliteal nodes) may be the only presenting sign, termed an incomplete primary complex [14,33,34].

Pulmonary lesions do occur in cats, but rarely, and result from bacteria being inhaled and causing typical tubercle formation in the lungs and hilar lymph nodes [9,14]. Much more commonly, pulmonary disease is secondary to the putative haematogenous spread of bacteria from the site of inoculation in the skin [12,14]. This generates a diffuse interstitial pattern of disease which eventually becomes bronchial and is clinically observable as progressive dyspnoea followed eventually by a soft productive cough [14,34]. Radiographically this presents interstitial patterd differs from primary pulmonary infection which more frequently causes the "classical" tuberculous cavitating lesions [34]. Disseminated disease can cause a range of clinical signs including hepato-splenomegaly, pleural and pericardial effusions, generalised lymphadenopathy, weight loss and pyrexia [9,13,33,34,42].

With such variable clinical presentation and non-specific clinical signs, diagnosing feline mycobacterial infections rapidly and accurately is challenging. Mycobacterial culture conducted by the Animal and Plant Health Agency (APHA), is currently the recognised 'gold standard test' for the diagnosis of mycobacterial disease in UK companion animals; however, it has several limiting features. Firstly, it has been shown to have a relatively poor sensitivity of ~50%, secondly it requires weeks from the time that a contaminated tissue biopsy is submitted for results to be obtained *e.g.* the average culture time is 12-16 weeks for *M. microti*

- the most frequently cultured organism [9,34,43]. During this time, if untreated, a patient will remain a potential source of infection to both its owner and the local environment.

As such, alternative diagnostic tests have been developed which aim to reduce the amount of time between clinical presentation and a definitive diagnosis being reached. Interferon-gamma (IFN-γ) release assays (IGRA) were developed on the principle of quantitatively evaluating IFN-γ production by peripherally circulating antigen-specific effector T-memory cells upon *in vitro* stimulation, in order to aid the diagnosis of bTB in cattle [44–47]. These have subsequently been adapted to identify both active and latent tuberculosis in human patients with both greater sensitivity and specificity than the previously utilised tuberculin skin test [48–50]. Where intradermal testing has been shown to be of unreliable clinical utility in the cat, the IGRA has been used successfully [14,46].

The IGRA tests have several advantages over other diagnostic techniques; they are significantly quicker at generating results than culture and are cheaper than many commercially available PCR and subsequent sequencing methods, they are also relatively non-invasive requiring only a single peripheral blood sample [46,49]. Unlike intradermal skin testing, they can be repeated if necessary as conducting the assay does not alter the systemic immune response [45,51,52]. In 2008, an adapted methodology of the IGRA was validated for diagnostic use in cats; this assay has a reported 100% sensitivity to indicate MTBC infection [46].

Recently, within the field of human diagnostics of mycobacterial diseases, and of tuberculosis in particular, there has been a marked increase in research focussed on the identification of circulating cytokine biomarkers for the diagnosis of both active and latent MTBC infections in humans [53–58]. The goal of such assays is to cheaply, sensitively and rapidly identify infected and infectious individuals, in order to combat what remains one of the most incident infectious diseases of man [59]. Such studies have utilised multiplex cytokine assays, most commonly Luminex xMAP technologies, with promising results [55,60,61]. Studies of multiple assays have found circulating cytokine concentrations and combinations of cytokines which can sensitively and specifically diagnose both active and latent tuberculosis in humans [53–58]. For example;

CXCL10 and CCL2 plasma concentrations can be combined with circulating levels of IFN-γ to more accurately discriminate between active and latent TB states, whilst the quantification of vascular endothelial growth factor can help differentiate patients with tuberculous pleural effusion from those with neoplastic pleural effusion [58,62]. Similar assays have also been used successfully in the study of a limited number of companion animal diseases, including canine lymphoma and feline cystitis [63–66].

As the timely and accurate diagnosis of feline mycobacteriosis, and the identification of the causative species is currently challenging, the aim of this study was to evaluate whether cytokine profiling from peripheral blood samples of infected cats demonstrate the same clinical utility as has been shown for humans. We therefore hypothesised that a number of cytokines would be differentially detectable in cats infected with mycobacterial infections when compared to both healthy controls and sick cats hospitalised for other reasons. We further hypothesised that the cytokine response would differ according to the species of mycobacteria present in the patient.

3.2 Materials and Methods

3.2.1 Blood Sample Collection from *Mycobacteria* spp. Infected Cats

Blood samples analysed in this study were either heparinised plasma or separated serum. Archived remnant samples obtained opportunistically by vets and donated by the cats' owners were used in this project. Cats diagnosed with mycobacterial infection by histological identification of (pyo)granulomatous inflammation in lesion biopsy material with the presence of AFB morphologically indicative of mycobacteria were considered eligible for inclusion in this study. Private veterinary surgeons who contacted the Royal (Dick) School of Veterinary Studies (RDSVS) for clinical assistance with case management were asked to retain any blood samples remaining after diagnostic procedures had been performed. With the owner's consent, these samples were then sent directly to the RDSVS where they were retained frozen prior to the analysis of chemokine and cytokine concentrations.

Cats were excluded from the study if, prior to blood sample collection, they had been known to be treated with immunomodulatory medications e.g. non-steroidal anti-inflammatory drugs (NSAIDs), chemotherapeutic agents or corticosteroids within 14 days of sample collection. Cats were additionally excluded if they were pre-treated with antibiotics with efficacy against mycobacteria, including fluoroquinolones, macrolides/azides or doxycycline within the same time period. Cats were not excluded if they had been treated with antimicrobial agents if these were likely to be ineffective against mycobacteria, such as a penicillin or cephalosporin. Similarly, cats were not excluded if they had been treated with non-immunomodulatory analgesic medications e.g. opioids.

3.2.2 Speciation of Mycobacteria Infecting Case Animals

All 116 cats included in this study had positive histological diagnoses of mycobacterial infection as outlined above (see Section 2.2.1). Of these, cases which had undergone additional IGRA, PCR or culture testing were sub-classified by the species of infecting organism identified.

Cases were defined as being infected with an MTBC mycobacteria if the cat had an IGRA test which showed an antigen specific IFN-γ response biased to purified protein derived from *M. bovis* (PPDB) above that generated in response to purified protein derived from *M. avium* (PPDA). The MTBC cases which additionally responded to the ESAT-6/CFP-10 peptide combination were classified as *M. bovis* infected. Cats which did not respond to the ESAT-6/CFP-10 peptide cocktail but showed a PPDB biased response were defined as being infected with *M. microti.* Cats with a positive IGRA test that did not meet the criteria for MTBC infection were classified as being infected with NTM. Both the test method and interpretation have previously been described by Rhodes *et al.* (2008).

In addition to IGRA testing, where available the results of specialist mycobacterial culture conducted at Weybridge reference laboratory by the APHA from tissue biopsies were used to speciate infections. In some cases, PCR results were used to speciate infections: these were conducted by Leeds University Mycobacterial Reference Laboratory (LUMRL). In total, 80 of the 116 cats tested could have the infecting species of mycobacteria identified.

3.2.3 Collection of Control Cat Blood Samples

Remnant serum samples from 16 healthy control cats were kindly gifted from the Waltham Centre for Pet Nutrition. At the time of sampling each control animal underwent its annual routine health check comprising compete physical examination by the attending veterinary surgeon, plus full haematological testing and serum biochemical analyses. All cats were required to have been found to be healthy and their blood test results to be unremarkable to be included as control animals in this study. Separated serum were aliquoted, sent directly to the RDSVS and stored -80°C prior to analysis of chemokine and cytokine concentrations.

3.2.4 Collection of Hospitalised Cat Blood Samples

Remnant serum was obtained from six cats under treatment at the RDSVS. These cats had no clinical signs consistent with mycobacterial disease and were only included if an alternative definitive diagnosis had been reached. Once obtained, samples were kept at 4°C and assayed within 12 hours.

Cats were excluded from this group for any one or more of the following: a diagnosis of stage V neoplasia (*i.e*. bone marrow involvement was confirmed), myelodysplastic disorder, exogenous retroviral (FIV/FeLV) infection, or if they had received treatment with any immunomodulatory medicines including NSAIDs, corticosteroids or chemotherapeutic agents.

3.2.5 Cytokine and Chemokine Measurements

Cytokine and chemokine concentrations were measured in all of the samples using a commercial, feline specific, antibody-coated microsphere-based multiplex cytokine immunoassay shown to be able to quantify 19 cytokines contemporaneously using 25µL of each patient's serum (FCTYOMAG-20K MILLIPLEX MAP Feline Cytokine/Chemokine Magnetic Bead Panel, Premix 19 Plex Kit, MERCK Millipore Corporation, Billerica, MA, USA).

The cytokines measured were; cluster of differentiation (CD) 95 (also known as sFas), Fms-related tyrosine kinase three (Flt-3) ligand, granulocyte-macrophage colony-stimulating factor

(GM-CSF), IFN-γ, interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-12-(p40), IL-13, IL-18, keratinocyte chemoattractant (KC), monocyte chemoattractant protein (MCP)-1, platelet derived growth factor dimeric for subunit β (PDGF-BB), C-C motif ligand 5 (CCL-5, also known as 'regulated on activation, normal T-cell expressed and secreted' or RANTES), stem cell factor (SCF), stromal-cell derived factor (SDF)-1 and tumour necrosis factor (TNF)-α.

All reagents as well as the serum/plasma samples were initially brought to room temperature. The pre-mixed bead solution was sonicated for 30 seconds and then vortexed for one minute. Quality Control vials 1 and 2 (FCTYOMAG-20K Kit) were each reconstituted with 250µL of deionised water. Serum Matrix (FCTYOMAG-20K Kit) was made by the addition of 1mL of deionised water and lyophilised Serum Matrix which was allowed to reconstitute at room temperature for ten minutes before a further 2mL of Assay Buffer (FCTYOMAG-20K Kit) was added. Patient and control animal sera were diluted in a 1:3 in Serum Matrix before analysis. The standard curve panel was produced by conducting a seven step 4-fold dilution series of the Feline Cytokine Panel Standard (FCTYOMAG-20K Kit) in Assay Buffer following reconstitution in 250µL of deionised water. Each assay plate was initially coated with 200µL/well of Assay Buffer which was left for ten minutes on a plate shaker prior to the Buffer being removed.

An aliquot of 25µL of appropriate Standard, Quality Control or diluted serum sample was added to each well as appropriate; all samples were assayed in duplicate and mean values analysed. An additional 25µL of Assay Buffer was added to the 0pg/mL standard (background) wells and to each well containing test serum, whilst the same volume of serum matrix was added to the Standard and Control wells. Finally, 25µL of the pre-mixed bead solution was added to each well before the plate was covered with aluminium foil and incubated overnight (16 hours) at 4^{o}C on a plate shaker.

The following day, the contents of each well was removed and each well was washed twice with 200µL of Wash Buffer. The Detection Antibody solution (FCTYOMAG-20K Kit) was allowed to warm to room temperature before 25µL was added to each well. The plates were sealed, covered with aluminium foil and incubated at room temperature on a plate shaker for

an hour; 25µL of streptavidin-phycoerythrin (FCTYOMAG-20K Kit) was then added to each well and the plate was incubated at room temperature on a plate shaker for an additional half an hour. The contents of each well was removed and each well was washed twice with 200µL of Wash Buffer (FCTYOMAG-20K Kit).

The plates were read with a multiplex plate reader and companion software (Luminex 200). All cytokine and chemokine concentrations were calculated from the median fluorescent intensity (MFI) prior to statistical analysis and the results are therefore reported in pg/mL.

3.2.6 Statistical Analysis

Statistical analysis was performed using commercially available statistical software (GraphPad Prism 7.0). Mean MFI values of duplicate samples for each cat were used for analysis. For the purposes of statistical analysis, values that fell below the limit of detection of the assay were assigned a concentration of 0 pg/mL.

Initially, to compare all mycobacteria positive samples (n=116) to healthy control cats (n=16), the D'Agostino & Pearson omnibus test was used to confirm a Gaussian distribution of the control group data for each of the cytokines/chemokines measured. As no reference intervals currently exist for these biomarkers in the cat, these were generated from the healthy control cat population to include values 1.96 standard deviations above and below the mean of the control group (*i.e.* to encompass ~95% of the healthy population) for each cytokine/chemokine measured. The mean concentration, standard deviation and reference interval (RI) generated for each molecule measured.

For mycobacteria-infected cats, 95% confidence intervals (CI) around the mean were generated by logarithmic-transformation of the cytokine values so that the data conformed to a Gaussian distribution. On this basis, a 95% confidence interval was then generated for the transformed data which was then reverse transformed to give the confidence intervals provided in Table 3.1.

Cytokine concentrations were considered to be statistically significantly different between mycobacteria-infected and healthy control cats if there was no overlap between the RI generated from the control data and CI of data generated from infected cats (shown in bold in Table 3.1).

A second comparison was made between mycobacteria-infected, healthy control cats and hospitalised cats. Confidence intervals for the cytokine levels of hospitalised cats were generated in the same way as described for mycobacteria-infected cats and are shown in Table 3.1. Again, cytokine levels were considered to be significantly different between groups if there was no overlap between the RI generated from the control data and CI of the concentrations measured in serum from sick cats or mycobacteria infected cats.

A sub-group analysis was conducted to compare healthy control (n=16), *M. bovis* infected (n=22), *M. microti* infected (n=43) and NTM infected cats (n=15) with each other. Of these four groups, only cytokine concentrations within the healthy control group followed a Gaussian distribution as assessed by D'Agostino & Pearson omnibus test. Therefore, a Kruskal-Wallis test by ranks was used to determine if differences existed between the groups for each cytokine measured. Where significant differences were found (defined as $p<0.05$), each group was compared in a consecutive pairwise manner using Mann-Whitney U tests with *post-hoc* Bonferroni correction applied to accommodate for multiple comparisons. This resulted in a value of $p\leq0.003$ being considered statistically significant for sub-group analyses. The median, range and 95% confidence interval for the cytokine concentrations measured within each sub-group of mycobacteria-infected cats are shown in Table 3.2.

3.3 Results

Table 3.1: Cytokine concentrations detected for each group of cats; healthy controls, cats infected with *Mycobacteria spp.* and cats hospitalised for other reasons. All concentrations are given in pg/ml. Rows in bold denote a statistically significant difference between at least two of the three groups. Legend: N; total number of cats per group, SD; standard deviation.

Cytokine/ chemokine	**Control group (N=16)** Mean ± SD (Reference interval)	**Mycobacteria infected (N=116)** Median; range (95% confidence interval)	**Hospitalised cats (N=6)** Median; range (95% confidence interval)
sFAS	**33.09 ± 18.16 (24.18-41.89)**	**12.25; 0-191.92 (5.32-19.06)**	**32.17; 23.06-45.35 (28.73-47.07)**
Flt-3L	43.34 ± 15.37 (35.81-50.87)	69.20; 20.76-223.63 (41.46-77.59)	49.74 (2.44-473.41)
GM-CSF	**11.53 ± 4.65 (9.25-13.81)**	**24.82; 0-60.17 (19.19-29.37)**	**10.20; 0-13.72 (4.81-17.85)**
IFN-γ	71.59 ± 55.17 (44.55-98.62)	76.93; 2.22-5499.63 (0-220.14)	125.45; 34.29-300.30 (12.06-612.74)
IL-1β	**28.79 ± 9.09 (24.33-33.25)**	**18.97; 0-272.72 (15.74-32.19)**	**5.00; 0-7.83 (1.23-15.67)**
IL-2	**0 ± 0 (0-0)**	**86.62; 0-1129.91 (22.65-135.28)**	**0; 0-0 (0-0)**
PDGF-BB	**0 ± 0 (0-0)**	**450.33; 0-1323.25 (371.34-529.31)**	**214.55; 0-267.54 (128.14-344.52)**
IL-12 (p40)	157.16 ± 93.26 (111.47-202.86)	359.62; 77.03-236.74 (100.91-460.51)	358.32; 64.19-643.79 (53.01-1494.22)
IL-13	**44.40 ± 18.28 (35.44-53.36)**	**20.05; 2.29-118.58 (14.99-25.89)**	**7.17; 0-8.65 (1.74-12.97)**
IL-4	**212.03 ± 183.34 (122.19-301.87)**	**9.38; 2.65-68.58 (3.82-14.90)**	**568.28; 74.05-2500.93 (38.95-6357.42)**
IL-6	65.84 ± 47.44 (42.59-89.10)	429.86; 1.51-1938.58 (0-1184.39)	37.31; 3.94-79.67 (3.57-208.10)
IL-8	**16.10 ± 17.61 (7.48-24.73)**	**137.80; 14.72-3644.62 (46.21-346.12)**	**50.95; 2.18-267.69 (4.55-232.43)**
KC	**3.18 ± 1.58 (2.41-3.95)**	**16.10; 1.21-624.47 (11.40-32.31)**	**125.99; 2.12-186.32 (0.00-2588.71)**
SDF-1	658.01 ± 265.07 (528.13-787.89)	332.31; 39.34-2671.33 (50.68-705.69)	304.70; 0-428.28 (122.73-671.10)
RANTES	**9.45 ± 3.72 (7.62-11.28)**	**19.28; 2.41-505.13 (16.21-51.97)**	**5.68; 0.23-5.36 (2.21-13.44)**
SCF	74.03 ± 19.89 (64.28-83.77)	75.14; 9.58-827.30 (53.14-106.28)	66.27; 5.69-205.36 (32.21-151.14)
MCP-1	1497.87 ± 779.95 (1115.69-1880.04)	1297.77; 37.62-13449.58 (341.74-1320.36)	1348.69; 560.01-2142.26 (445.38-3369.35)
TNF-α	**34.41 ± 15.07 (27.02-41.79)**	**54.84; 10.57-1527.12 (51.25-128.27)**	**16.42; 0-18.82 (12.20-21.78)**
IL-18	200.80 ± 194.17 (105.66-295.94)	133.40; 0-1932.34 (0-108.39)	756.99; 539.00-974.28 (411.54-1314.89)

Table 3.2: Cytokine concentrations detected for each sub-group of cats infected with *Mycobacteria spp.* designated into those cases infected with *M. bovis, M. microti* or non-tuberculous (NTM) mycobacteria. All concentrations are given in pg/ml. Rows in bold denote a statistically significant difference (P≤0.003) between at least one of these groups and the healthy control cats (Table I). Legend: N; total number of cats per group.

Cytokine/ chemokine	***M. bovis*-infected cats N=22** Median (95% confidence interval)	***M. microti*-infected cats N=43** Median (95% confidence interval)	**NTM infected cats N=15** Median (95% confidence interval)
sFAS	8.26 (4.30-13.79)	7.28 (2.28-12.58)	15.70 (12.02-23.79)
Flt-3L	**80.79 (62.69-90.61)**	**73.54 (64.47-101.37)**	**42.87 (31.80-54.62)**
GM-CSF	**27.63 (20.92-31.74)**	**27.88 (20.85-31.78)**	**11.25 (10.87-11.57)**
IFN-γ	89.74 (35.18-111.13)	270.21 (79.63-337.83)	52.58 (28.95-89.39)
IL-1β	24.52 (21.69-26.28)	27.93 (25.60-31.48)	27.87 (27.14-76.00)
IL-2	**132.59 (115.52-155.35)**	**176.97 (149.59-185.48)**	**14.08 (2.04-17.07)**
PDGF-BB	**0 (0-0)**	**363.47 (148.47-511.47)**	**0 (0-0)**
IL-12 (p40)	**309.53 (289.67-646.77)**	**457.38 (332.72-550.28)**	**283.89 (200.40-486.63)**
IL-13	40.74 (21.08-70.01)	28.42 (23.87-59.31)	14.16 (2.24-112.20)
IL-4	55.89 (23.66-146.82)	154.55 (86.89-355.49)	76.49 (20.08-137.54)
IL-6	**15.91 (5.65-28.98)**	**33.98 (20.51-99.72)**	**29.64 (9.08-64.30)**
IL-8	**105.84 (59.86-215.20)**	**164.68 (51.89-201.06)**	**500.67 (137.47-818.24)**
KC	9.18 (2.39-20.12)	15.16 (2.33-19.75)	35.79 (1.88-97.68)
SDF-1	**2.88 (2.00-26.89)**	**20.41 (2.81-26.54)**	**20.28 (4.59-94.51)**
RANTES	**43.18 (23.92-58.29)**	**27.18 (24.94-59.96)**	**45.24 (9.94-71.63)**
SCF	73.87 (56.86-103.04)	192.57 (90.03-208.47)	69.25 (43.43-90.25)
MCP-1	**472.40 (202.24-1946.22)**	**1136.32 (441.82-2398.05)**	**70.77 (54.21-193.52)**
TNF-α	**162.47 (155.53-194.18)**	**41.58 (18.02-55.98)**	**12.84 (11.07-39.67)**
IL-18	117.04 (114.00-222.90)	45.62 (39.06-166.44)	38.05 (22.97-147.84)

3.3.1 Patient Characteristics

All cats included in this study were adult cats from the UK. In total, serum/plasma from 116 cases of feline mycobacterial disease met the inclusion criteria for the study, and serum from 16 healthy control cats and six hospitalised cats was also analysed.

Within the group of cats with clinical mycobacteriosis, the infecting organism was speciated based on IGRA, culture and/or PCR for 80 of the 116 (69.0%) cats. Of these, 22 (27.5%) were infected with *M. bovis*, 43 (53.8%) were infected with *M. microti* and the remaining 15 (18.7%) infected with NTM.

For the cats hospitalised for other diseases the final diagnoses were established as hyperthyroidism (n=2), congestive heart failure due to hypertrophic cardiomyopathy (n=1), hepatic lipidosis (n=1), lysosomal storage disease (n=1), chronic kidney disease (n=1) and diabetes mellitus (n=1).

3.3.2 Cytokine and Chemokine Concentrations

All mycobacteria-infected cats compared to healthy control cats

The mean/median and standard deviation/range for each cytokine concentration for each of these groups is shown in Table 3.1. Three cytokines; sFAS, IL-13 and IL-4 were found to be significantly reduced in the mycobacteria-infected group compared to healthy control cats (Figure 3.1). By contrast, seven cytokines; GM-CSF, IL-2, PDGF-BB, IL-8, KC, RANTES and TNF-α were present in significantly elevated concentrations within the peripheral circulation of mycobacteria infected cats when compared to healthy control animals (Figure 3.2).

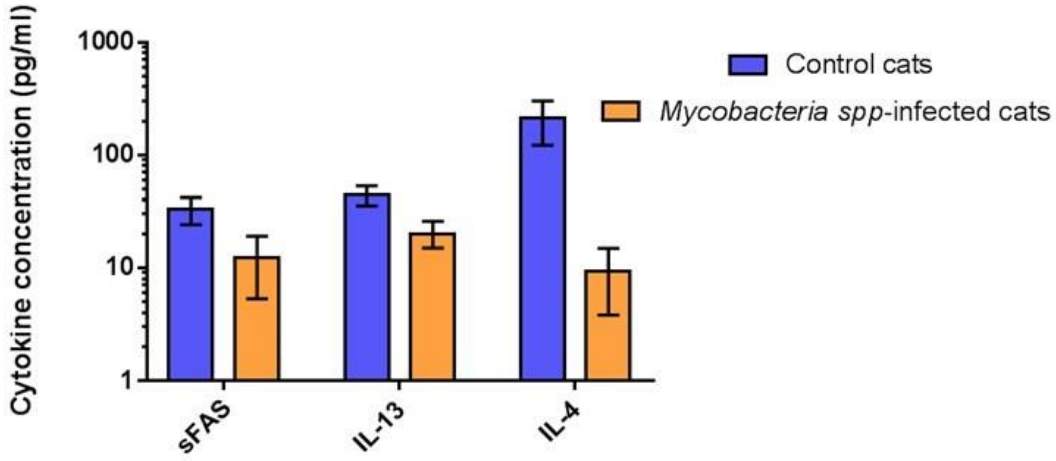

Figure 3.1: Cytokines found to have significantly reduced concentrations in the peripheral blood of cats with mycobacterial disease compared to healthy controls. Data are shown as the median for the group with error bars indicting the 95% confidence interval.

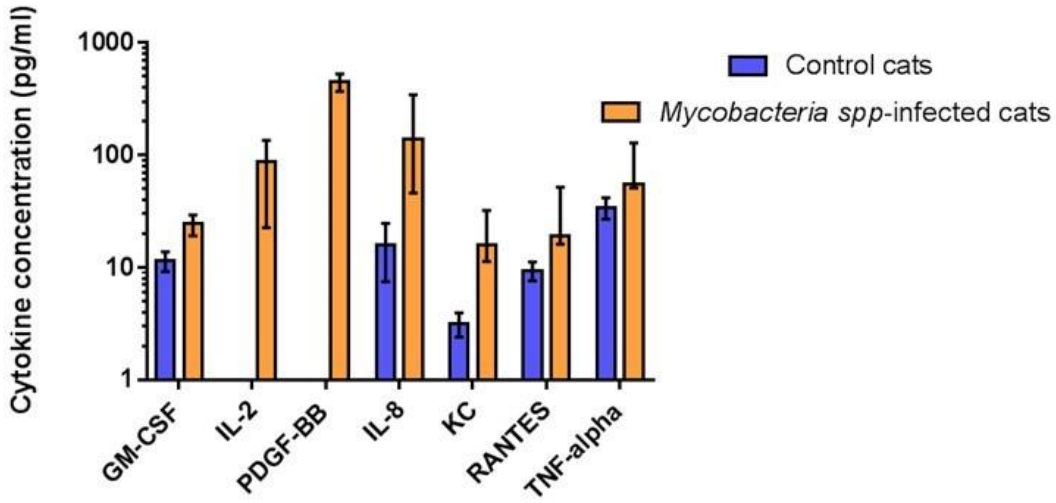

Figure 3.2: Cytokines found to have significantly increased concentrations in the peripheral blood of cats with mycobacterial disease compared to healthy controls. Data are shown as the median for the group with error bars indicting the 95% confidence interval.

There was no significant difference between the two groups in their circulating concentrations of Flt-3 ligand, IFN-γ, IL-1β, IL-12 (p40), IL-6, IL-18, SDF-1, MCP-1, or SCF.

Comparison of all mycobacteria-infected cats to hospitalised cats and controls

To begin to assess the specificity of changes in cytokine concentrations between feline mycobacteriosis patients and healthy controls, serum from six hospitalised cats was analysed and compared to both of these groups, data shown in Table 3.1.

The cytokines GM-CSF, IL-2, PDGF-BB, RANTES and TNF-α were found at significantly elevated concentrations in mycobacteria infected cats, whilst IL-4 and sFAS were detected at reduced concentrations in mycobacteria infected cats compared to both hospitalised non mycobacteria-infected cats and healthy controls (Figure 3.3).

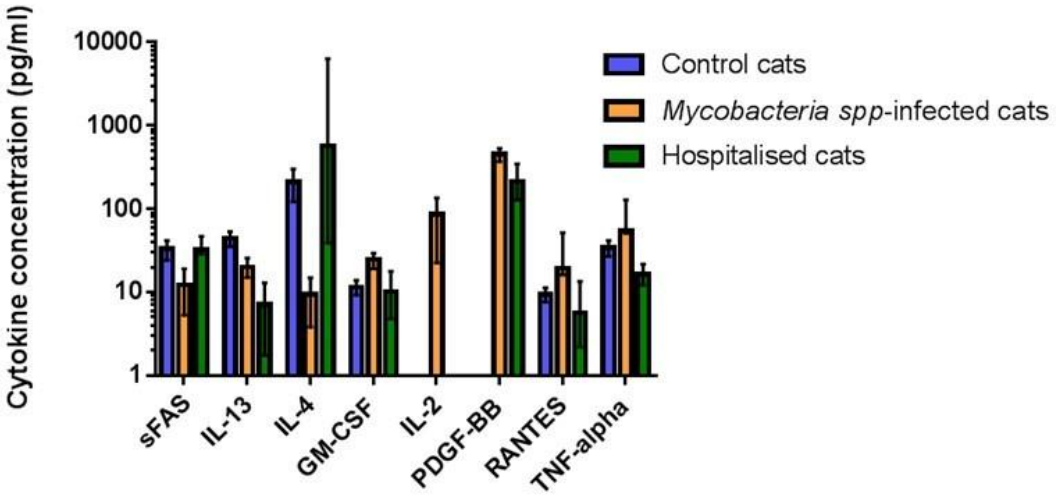

Figure 3.3: Cytokines found to have significantly altered concentrations in the peripheral blood of cats with mycobacterial disease compared to healthy controls and/or cats hospitalised for other reasons. Data are shown as the median for the group with error bars indicting the 95% confidence interval.

The concentrations of IL-13 and IL-1β were found to be significantly reduced in hospitalised cats when compared to both control cats and those with mycobacterial infections (Figure 3.4). Whilst differences in the concentrations of IL-8 and KC were observed between healthy cats and those infected with mycobacteria, there was no difference in the concentrations of these cytokines between cats infected with mycobacteria and cats hospitalised for unrelated conditions (Figure 3.4).

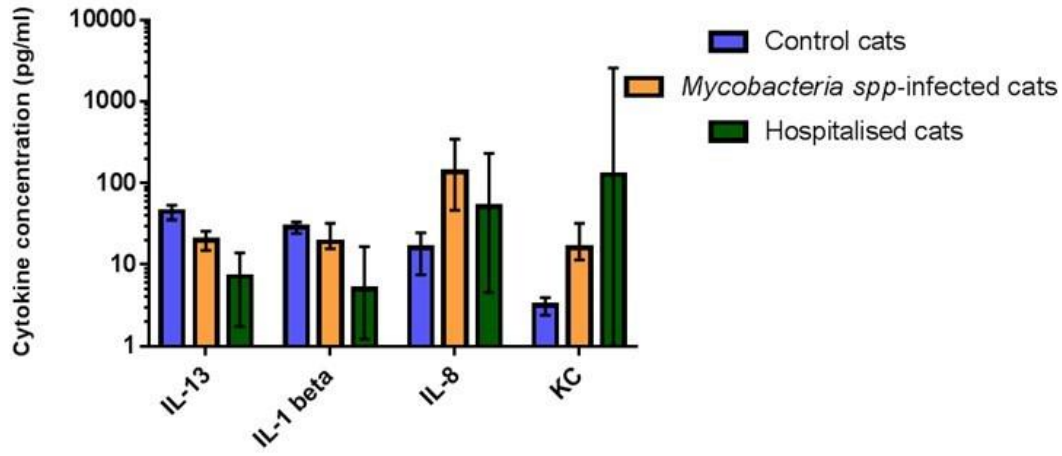

Figure 3.4: Cytokines found to have significantly altered concentrations in the peripheral blood of cats with mycobacterial disease compared to healthy controls and/or cats hospitalised for other reasons. Statistically significant differences between groups were determined by Kruskal-Wallis test by ranks ($P<0.05$), each group was compared in a consecutive pairwise manner using Mann-Whitney U tests with post-hoc Bonferroni correction applied to accommodate for multiple comparisons ($P\leq0.003$). Data are shown as the median for the group with error bars indicting the 95% confidence interval.

Comparison of cats infected with different mycobacterial species

Of the 116 cats enrolled in the study, 80 had their mycobacterial infections definitively speciated to one of three causative agents, or group of causative agents; *M. bovis, M. microti* and NTM infections. These groups were compared to one another and also against the healthy control group. This analysis revealed the concentration of TNF-α to be significantly increased in the *M. bovis*-infected group when compared to all other groups; while the concentration of PDGF-BB was found to be significantly increased in cats infected with *M. microti* compared to all remaining groups (Figure 3.5). Concentrations of GM-CSF, IL-2 and Flt3-L were significantly increased in both MTBC groups (*M. bovis* and *M. microti*) when compared to the remaining two groups (Figure 3.6).

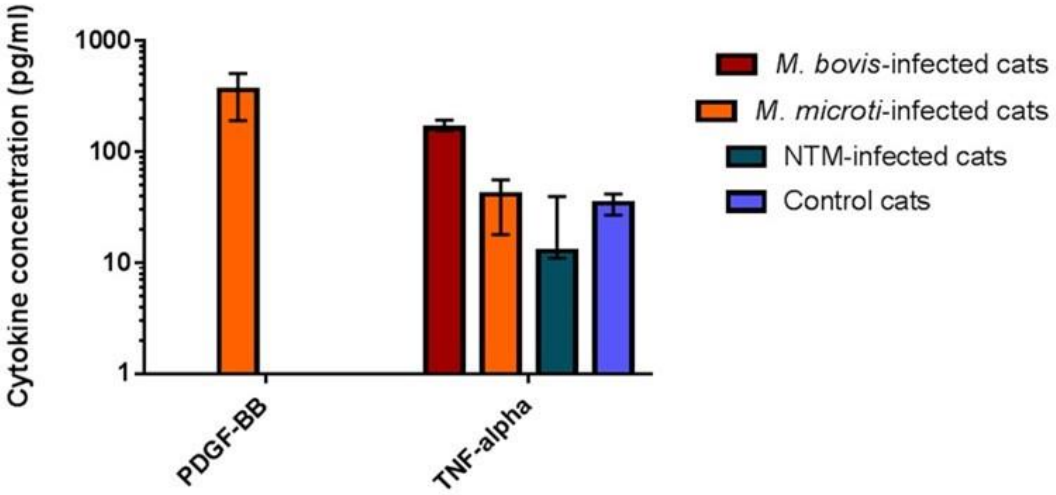

Figure 3.5: Cytokines found to have significantly altered concentrations in the peripheral blood of cats with mycobacterial disease due to *M. bovis* or *M. microti,* respectively, compared to healthy controls and cats infected with non-tuberculous mycobacterial (NTM) species. Data are shown as the median for the group with error bars indicting the 95% confidence interval.

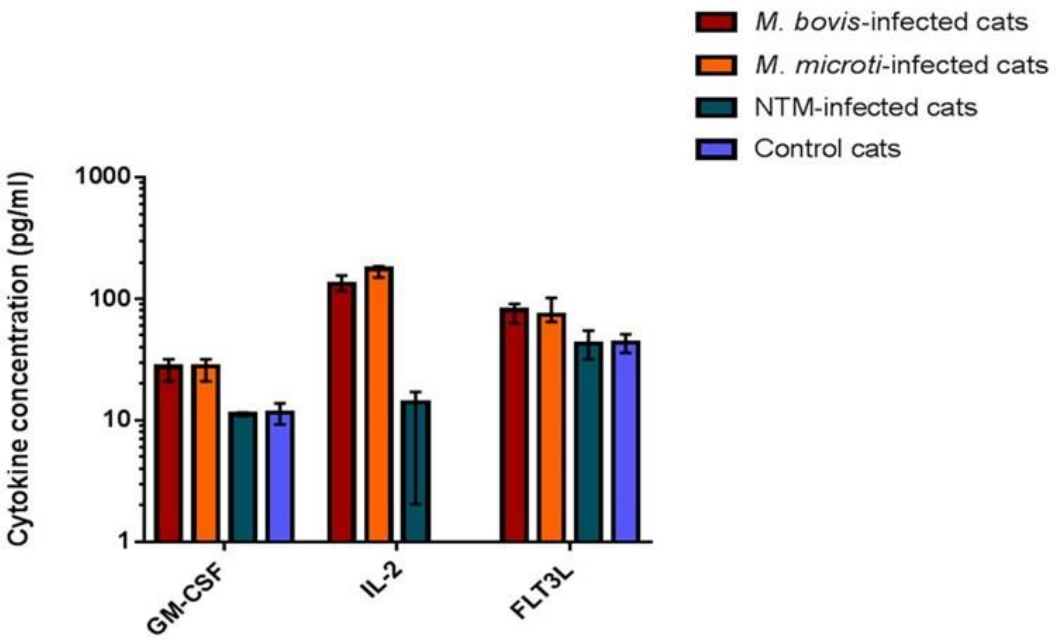

Figure 3.6: Cytokines found to have significantly altered concentrations in the peripheral blood of cats with mycobacterial disease due to TB-complex infections (*M. bovis* or *M. microti* respectively), compared to healthy controls and cats infected with non-tuberculous mycobacterial (NTM) species. Data are shown as the median for the group with error bars indicting the 95% confidence interval.

The group of cats infected with NTM species had a significant reduction in the concentration of MCP-1 in comparison to the healthy controls (data not shown).

Concentrations of cytokine IL-8 were increased in all three mycobacteria-infected groups when compared to healthy control animals (data not shown), with the greatest increase observed in the cats with NTM infections, though this trend was not statistically significant (p=0.07).

3.4 Discussion

Current routine clinical methods for the diagnosis of feline mycobacterial disease, and tuberculosis in particular, have a number of drawbacks. Multiplex immunoassays have proven to be sensitive, specific, rapid, require very small sample volumes, and show a broad analytical and dynamic range [67-71]. The benefits of the multiplex assay approach used in this study include more rapid availability of results than is currently achievable with culture or IGRA, and that this analysis requires only a small volume of serum or plasma to analyse (25uL) [67–71].

This population of cats consisted of adult cats resident in the UK; previous studies have shown that male cats are over-represented in the population of cats presenting with mycobacterial infections and cats are usually younger when they present with *M. bovis* compared to *M. microti* infections [13,34]. It was not possible for us to determine if any such bias existed within our study population as signalment data was infrequently reported to us by primary veterinary clinicians. The reasons for this are unclear but it may be a reflection of the predominately outdoor and semi-feral lifestyles of many of these cats meaning that there is limited information definitively known about them.

All samples used in this study were opportunistically obtained remnants collected as part of other diagnostic investigations and processed by primary veterinary clinicians. This approach meant that samples could be sourced ethically and provides demonstrable ‘proof of concept’ that such methodology is potentially practicable and achievable within a clinical setting. Due to the remnant nature of samples analysed in this study, one must be cognisant of the possibility that post-collection processing may not have been consistent across samples e.g. storage temperature, which may have influence on individual findings [72,73]. However, it seems reasonable to presume that such adverse effects would not impact any one group of cats in this study in particular, but occur randomly throughout the dataset generated and so the results

obtained in this study are still (clinically) interpretable. Similarly, due to the opportunistic nature of sample collection, this study included two different types of sample media; serum and heparinised plasma. Previous work with the same commercially available multiplex assay kit has demonstrated strong correlation between cytokine/chemokine concentrations obtained in paired serum and heparinised plasma samples taken from the same cat [71,74], and so again it would seem the results obtained in this study remain reliable and interpretable.

The inflammatory cascade induced by mycobacterial infection is complex and is poorly characterised in the cat. The hallmark lesion of mycobacterial infections, including TB, is the granuloma which is a compact, organised aggregate of immune cells formed as part of the host immune response to the persistent stimuli of the pathogen [15,75–77]. Upon phagocytosis, inhibition of phagosome-lysosome fusion enables the intracellular survival of mycobacteria within these phagocytes [78]. The internalised bacilli are then believed to stimulate the infected macrophage to invade the local tissues [79,80]. The stimulation of macrophage Toll-like receptors (TLR) by mycobacteria induces the production of numerous cytokines, predominantly TNF-α, which drives the recruitment of mononuclear cells and neutrophils from surrounding blood vessels. In particular, there is recruitment of activated T-cells which interact with activated macrophages and secrete IFN-γ leading to mycobacterial killing [81–85]. Additionally, each group of recruited cells also release their own assortment of cytokines and chemokines which perpetuate the inflammatory cascade and the formation of a stable granuloma structure [82,86].

In this study, we demonstrated that changes in the concentrations of eight critically important cytokines gave a sensitive and specific indication of mycobacterial infection in this feline population. Elevated concentrations of the cytokines GM-CSF, IL-2, PDGF-BB, IL-8, KC, RANTES and TNF-α in mycobacteria infected cats with concurrent reductions in sFAS, IL-13 and IL-4 are suggestive of a pro-inflammatory process occurring in these patients, which is dominated by the recruitment and maturation of monocyte-macrophage lineage cells, the recruitment of cytotoxic T-cells, the proliferation of fibroblasts and T-cells and the suppression of humoral immunity.

Granulocyte-macrophage colony-stimulating-factor (GM-CSF) is a critically important cytokine involved in the innate immune response against mycobacteria, specifically via influencing macrophage and dendritic cell function [87–93]. GM-CSF deficient mice are extremely susceptible to mycobacterial disease and their macrophages fail to effectively inhibit intracellular pathogen replication [91]. Similarly, humans with detectable titres of anti-GM-CSF auto-antibodies show an increased susceptibility to pulmonary tuberculosis compared to healthy controls, whilst GM-CSF immunotherapy can contribute to the resolution of disease due to drug-resistant *M. tuberculosis* [94,95]. GM-CSF activated cells, principally macrophages, go on to propagate the inflammatory cascade in particular via the production of TNF-α [87].

GM-CSF has been shown by previous multiplex cytokine analyses to be critical in the early response and possible clearance of *M. tuberculosis* in humans and mice, so that the use of recombinant GM-CSF as an adjunctive therapy for TB has been posited; the findings of this study suggest that this may also be of benefit to feline patients.

TNF-α is an agonist of both NF-kB and mitogen-activated protein kinase (MAPK) which lead to downstream upregulation of pro-inflammatory immune functions including but not limited to; increased cell adhesion (e.g. giant cell formation) and macrophage apoptosis which are beneficial to the control of mycobacterial infections [96–98]. One such downstream effect is the increased expression and secretion of chemokines including RANTES and interleukin IL-8 [99–101]. These molecules are major drivers in the recruitment and migration of leukocytes towards an inflammatory focus [99–101]. Additionally, RANTES can combine synergistically with other locally secreted cytokines, such as IL-2 released from T-cells and IL-12(p40) released from pathogen-activated antigen-presenting cells e.g. mature dendritic cells, in order to stimulate the activation and proliferation of natural killer (NK) cells, which ultimately leads to a local increase in mycobactericidal activity [99–101].

TNF-α, one of the most studied cytokines in the human mycobacterial response, has been repeatedly shown to be of significant diagnostic utility for mycobacterial infections in humans, with concentrations varying between TB and non-TB patients as well as being discriminatory between active and latent infections and generally being found to decline with successful

therapy [54–57]. Similarly, a number of patients receiving TNF-α antagonists such as infliximab have been reported to contract and even die from mycobacterial infections [102–104]. In this study, it is notable that this cytokine was the only one to be uniquely indicative of infection with *M. bovis.* This is potentially due to the secretion of RD-1 proteins during the course of these infections generating a more pro-inflammatory state in *M. bovis* infected cats. The possibility that cats infected with mycobacteria and with elevated TNF-α could have more severe or active disease and hence pose a more serious zoonotic threat to humans is one that warrants further investigation.

An essential step in the formation of an effective granuloma structure is fibroblast proliferation, mediated at least in part by the local production of PDGF-BB [105]. Similar to TNF-α, it has recently been demonstrated that circulating levels of this cytokine are elevated in human patients with active pulmonary tuberculosis, and that these levels decline significantly over a six month course of therapy [55]. However, in contrast to TNF-α, within our feline population, PDGF-BB was only detected at significant levels in cats infected with *M. microti.* This may indicate a difference in the immune response by cats to this species of mycobacteria, which was used as the human vaccine strain prior to the availability of *M. bovis*-BCG, and may therefore be considered attenuated [106]. Future analysis and comparison of the granuloma structures of *M. microti* and *M. bovis* infected cats may show physical manifestations of such differences, and may be useful to identify prognostic features of relevance to feline disease.

The Fas/Fas-ligand (FasL) system is an important molecule for immunological regulation; specifically it maintains T-cell homeostasis by the induction of apoptosis in order to limit T-cell expansion following antigenic stimulation [107,108]. It has been reported that mycobacteria exploit this system in order to subvert and evade the adaptive immune response of an infected animal by generating an immune-privileged niche [107,109]. Soluble Fas (sFAS) can antagonise cell surface FasL by competitive inhibition and so allow continued T-cell proliferation, a phenomenon shown to be significant in lymphoproliferative disorders such as non-Hodgkin's lymphoma [110].

This is the first time, to our knowledge, that evidence of such an extensively conserved systemic immune response to these infectious agents, directly comparable to that seen in many human and animal models of mycobacterial disease, has been demonstrated in the cat. This suggests that the occurrence of mycobacterial infections in this species could act as a naturally occurring model of human disease. This capacity could be particularly significant in overcoming the known shortcomings of murine models of mycobacterial disease, particularly as feline cases occur with relatively high clinical frequency and so could provide a naturally-occurring reliable source of study data.

Within the variety of mycobacterial species which are known to infect cats; those belonging to the MTBC, and *M. bovis* in particular, pose the greatest risk of zoonotic spread to in-contact humans [39–41]. Currently the gold standard diagnostic test to identify these organisms which can threaten human health is mycobacterial culture; however, *M. bovis* takes a minimum of six to eight weeks to confirm by culture and *M. microti* requires even longer at 12-16 weeks [9,14,43]. Within the cytokines analysed in this study; Flt-3L, GM-CSF and IL-2 were all significantly elevated in cats with a diagnosis of infection with an MTBC organism compared to all other groups. Therefore, it may be possible that these cytokines in particular could be used to more rapidly identify those infections that pose zoonotic risk to owners and the general public, allowing for earlier intervention.

Cytokine profiling in companion animal medicine has been the subject of a number of recent studies [63,65,66]. One limitation of such studies to date is the study design to compare cytokine profiles in animals with the designated diagnosis of interest and apparently healthy animals as controls. In our investigations, we additionally included a small number of cats that had been hospitalised for reasons unrelated to mycobacterial disease. This allowed us to identify that the changes in concentration of the cytokines KC and IL-8 were not specific to mycobacterial infection, but rather may simply occur when cats were ill or stressed. These findings indicate that including such a group in experimental design is and will be important for the assessment of diagnostic accuracy, including sensitivity and specificity, of cytokine profiling in the future. Greater accuracy would be achieved by expanding on our small sample size (n=6) and also

by matching the diagnoses in this group to those with similar aetiopathogenesis to the diagnosis of interest. For example, such a study could include cats with other infectious granulomatous disease such as feline infectious peritonitis (FIP), where multiplex expression profile analysis of serum samples have also shown elevations in RANTES and GM-CSF concentrations [65].

A limitation of the study we report here is its retrospective nature; this meant that it was not possible to have a standardised diagnostic assessment of each cat and samples from each cat did not undergo the gold standard test of mycobacterial culture. The majority of cats in this study were diagnosed by IGRA which has been shown to have a higher sensitivity for MTBC than NTM infections, which may have introduced some bias towards MTBC infected individuals in our population [46]. Similarly, the amount of time elapsed between the time of infection, the infective dose received, and the time of blood sampling was not known for these cats. It is therefore possible that the changes seen could reflect different stages or magnitude of the feline response to mycobacteria; for example, MTBC infections may be diagnosed earlier in the course of infection than NTM infections and so the differences seen may simply reflect an early compared to late immune response. It was therefore not possible for us to generate sensitivity or specificity data for these cytokines nor to assess their diagnostic capacity in combination as has been achieved in human medicine. To further investigate the potential for cytokine profiling in feline mycobacterial disease, the prospective recruitment of cases would allow for additional control over the time of blood sampling in relation to patients being presented to a veterinary surgeon. Such a study would additionally further allow changes in cytokine concentrations to be tracked over time, and in response to treatment. This would allow much greater accuracy regarding the cessation of antimicrobial therapy in these cases, which is not currently possible.

3.5 Conclusion

In this study, we demonstrate a conserved immunological response to mycobacteria by domestic cats analogous to that of other species. Further research into feline tuberculosis as a spontaneously occurring model of a significant human disease is now required. These data show that cytokine profiling has the practical and immunological potential to be developed as a sensitive and specific diagnostic test for the presence of mycobacteria in feline infections, to readily speciate them and help inform on zoonotic risk to exposed humans. The Milliplex MAP Feline Cytokine Magnetic Bead multiplex assay was shown to be a practicable and reliable platform for these measurements and further work should focus on the prospective diagnostic utility of the findings reported in this study.

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Chapter 4: A Feline Outbreak of *M. bovis* in the UK Associated with Feeding a Commercial Raw Food Diet.

Preface

The following Chapter presents the findings of a study, conducted as part of this studenship programme investigating an unprecedented outbreak of tuberculosis in domestic cats putatively due to the contamination of a raw pet food product. The findings are presented in four parts; the first three parts are presented as a chronological assembly of publications which document the outbreak and investigation in real time. Each publication contains a statement detailing the contribution of each author.

The fourth part of this Chapter comprises a detailed retrospective analysis of the outbreak; at the time of writing no new cases had been confrmed in the preceeding three months. Part four (O'Halloran, manuscript in preparation), joins together the epidemiological evidence established at the time of submission of this thesis with the clinical investigation findings to identify the most likely risk pathways by which the outbreak occurred; this would not have been possible without the assistance and resources of Tony Roberts of the Animal and Plant Health Agency who was instrumental in the epidemiological investigation.

Acknowledgments

Thanks go to all of the owners and veterinary surgeons of the cats in this study for donating samples and cadavers to us to allow the investigations to be conducted and for giving permission for the findings to be published.

PART 1

Mycobacterium bovis in pet cats

Veterinary Record, October 2018

Dear Editor,

We are writing with regard to three clinical cases of feline tuberculosis due to *Mycobacterium (M.) bovis* recently confirmed in two households in England. What makes these cases unusual is that these cases occurred in young pedigree cats (under two years old) with no history of outdoor access since they were adopted as kittens. Furthermore, both households are located well within areas of the country deemed to be low risk for *M. bovis* in cattle and other species including cats. These cases are therefore not typically consistent with how we currently think most cats become challenged with *M. bovis* – by hunting infected prey. Notably all cats were fed a commercial raw food diet but the significance of this in the epidemiology of these cases is not yet clear. Given the clinical particulars of these cases, it may be possible to prioritise differential diagnoses such as gastrointestinal lymphoma, or feline infectious peritonitis (FIP), but we would like to urge clinicians to be aware of the possibility of tuberculosis in young pedigree cats, even with a lack a history of outdoor access and that may have been fed on a raw food diet. If colleagues are suspicious of any cases, or have diagnosed any similar cases, we would be very interested in hearing from them.

Yours sincerely,

Conor O'Halloran & Danièlle Gunn-Moore; Royal (Dick) School of Veterinary Studies and The Roslin Institute, University of Edinburgh
Nicki Reed; Wear Referrals, Stockton-on-Tees
Julie Vickers; TDDS Ltd, Exeter
Eili Dettmering; Top Cat Veterinary Centre, Brighton
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Author contributions:

C. O'Halloran (the candidate), collated the case data and wrote the publication. Drs Reed, Vickers, Dettmering, Ioannidi and Murtagh provided case details. Professor Gunn-Moore supervised the project.

PART 2

Tuberculosis due to *Mycobacterium bovis* in pet cats associated with feeding a commercial raw food diet.

Journal of Feline Medicine and Surgery, April 2019

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Author contributions:

O'Halloran, C. conducted investigations, collated the clinical case details, supervised the clinical management of cases and wrote the manuscript.

Drs Ioannidi, Reed, Murtagh, Dettemering, Van Poucke, Gale and Howe diagnosed cases in practice, provided case details for publication and clinically managed cases in collaboration with the Univeristy of Edinburgh. Drs Vickers and Dobromylsky are experienced veterinary pathologists and provided diagnostic assistance for these cases. Dr Paul Burr, supervised the IGRA testing whilst Dr Gascoyne-Binzi conducted the PCR testing. Professors Hope and Gunn-Moore jointly supervised the investigations.

Abstract

Objectives: *Mycobacterium bovis,* a member of the *Mycobacterium tuberculosis*-complex*,* can infect cats and has proven zoonotic risks for owners. Infected cats typically present with a history of outdoor lifestyle and hunting behaviour, and cutaneous granulomas are most commonly observed. Six young cats, living exclusively indoors in five different households across England were presented to separate veterinarians across the UK with various clinical signs due to tuberculous disease.

Methods: Investigations into the pyogranulomatous lesions, lymphadenopathy and/or pulmonary disease of these cases consistently identified infection with *Mycobacterium bovis*. Infection was confirmed by PCR where possible or was indicated with a positive interferon gamma release assay (IGRA) where material for PCR was unavailable. In-contact, cohabiting cats were screened by IGRA and follow-up testing undertaken/advised where these were positive. A lifestyle investigation was undertaken to identify the source of infection.

Results: Six clinically sick cats and seven in-contacts have been identified with evidence of *M. bovis* infection. Five clinical cases were either too sick to treat or deteriorated despite therapy, giving a mortality rate of 83%. Lifestyle investigations revealed the common factors between clusters to be; that affected cats had mycobacterial infections speciated to *M. bovis*, were exclusively indoor cats and were fed a commercially available raw food product produced by a single manufacturer. The Food Standards Agency, Animal and Plant Health Agency, Public Health England and the food manufacturer concerned have been notified/informed.

Other possible sources of exposure for these cats to *M. bovis* were explored and were excluded; including wildlife contact, access to raw milk, the presence of rodent populations inside the buildings in which the cats lived, and exposure to known infectious humans.

Conclusions and relevance: Upon investigations, our results provide compelling, if circumstantial, evidence of an association between the commercial raw diet of these cats and their *M. bovis* infections.

4.1 Introduction

The increasing importance of mycobacterial infections in companion animals in the UK has become apparent in recent years[1,2]. It has been demonstrated that approximately 1% of all feline biopsies submitted for histopathological analysis show changes consistent with mycobacteriosis (pyo/granulomatous inflammation dominated by epithelioid macrophages) and at least one third of these have demonstrable Ziehl-Neelsen (ZN) positive organisms when stained, with a thin rod-like appearance, indicative of mycobacteria; these are also referred to as acid or alcohol fast bacilli (AFB)[3-5].

Clinically, about a third of feline mycobacteriosis cases in the UK are caused by *Mycobacterium tuberculosis*-complex (MTBC) pathogens, with *Mycobacterium (M.) microti* being cultured from 19% of all cases of feline mycobacteriosis, and a further 15% caused by *M. bovis*[6,7]*.*

There is a strong geographical predisposition to feline infections with members of the MTBC[6]. *M. bovis* infections are strongly co-incident with where there are high levels of endemic infection in local bovine and wildlife populations, such as the South-West of England[6-8]. Feline MTBC infections, particularly those caused by *M. bovis,* pose a potential zoonotic risk to their owners[9]; they may also act as a potential source of environmental contamination, which is critically important, as *M. bovis* is a pathogen of major animal health significance in the UK and other countries as it is the causative agent of bovine tuberculosis (bTB)[10].

In contrast to human tuberculosis, the majority of feline tuberculosis cases present with localised nodular cutaneous disease, frequently with a degree of ulceration and occasionally with draining sinus tracts[1,2,6,7,11,12]. The lesions are typically distributed around the face, extremities and tail base; the so-called “fight and bite sites” [1,2,6,7,11,12]. Skin lesions may be accompanied by a localised or occasionally generalised lymphadenopathy, or lymphadenopathy (usually of the submandibular, pre-scapular or popliteal lymph nodes) may be the only presenting sign, termed an incomplete primary complex[1,2,6,7,11,12].

Primary pulmonary lesions do occur in cats, but rarely[1,6]. They can result from bacteria being inhaled and causing tubercle formation in the lungs and hilar lymph nodes[1,2,6,7,11,12]. However, much more commonly, pulmonary disease is secondary to the putative haematogenous spread of bacteria from the site of inoculation in the skin[1,2,6,7,11,12]. This generates a diffuse interstitial pattern of disease that eventually becomes bronchial, and is clinically observable as progressive dyspnoea followed eventually by a productive cough[1,2,6,7,11,12]. Disseminated disease can cause a range of clinical signs including hepato-splenomegaly, pleural and pericardial effusions, generalised lymphadenopathy and weight loss[1,2,6,7,11,12]. Historically, tuberculosis in cats commonly presented as alimentary disease, caused by cats drinking tuberculous cow's milk, but following the introduction of milk pasteurisation, this form of tuberculosis is now only seen extremely rarely[1,2,6,7,11,12].

Due to the putative transmission of *M. bovis* and *M. microti* to cats by hunting infected prey, tuberculosis is most frequently diagnosed in adult cats, with a consistent history of hunting reported in almost every instance[6]. Male cats are over-represented, possibly secondary to contamination of fight-inflicted wounds[6]. The median age of infection is three years for *M. bovis* and eight years for *M. microti*[1,2,6,7,11,12]. There appears to be no link between MTBC infection and classical immunosuppression *i.e*. feline immunodeficiency virus (FIV) and feline leukaemia virus (FeLV) infection in cats[6].

The diagnosis of mycobacterial disease in cats is challenging; the traditional tuberculin skin testing technique has been shown to be ineffective in domestic cats[13]. Molecular techniques such as PCR and DNA hybridisation[1,14,15] are available through human Mycobacterial Reference Laboratories in the UK but remain expensive, and in some circumstances prohibitively so. These techniques also require the organism to be present, and are most sensitive when performed on a cultured isolate. Where this is not possible for a cat, then an IGRA is available[16,17]. This test uses specific mycobacterial proteins to stimulate peripheral immune cells in order to determine if a patient has been infected with an organism which either contains or secretes those peptides[16-18]. The production of interferon gamma in response to protein purified derived (PPD) from *M. bovis* (PPDB) at a greater concentration than that

produced in response to PPD from *M. avium* (PPDA), confirms infection with an MTBC organism[16,17]. A further peptide cocktail of the 6kDa early-secreted antigenic target (ESAT-6) and 10kDa culture filtrate protein (CFP-10) can be used to determine if a patient has been infected with an MTBC organism which encodes the genetic region RD-1; *M. tuberculosis* and *M. bovis* do encode this region, whilst *M. microti* does not[19,20]. Experimental challenge studies have shown that *M. tuberculosis* does not cause clinical disease in cats, so a positive response to ESAT-6/CFP-10 and PPDB in a feline patient indicates infection with *M. bovis*[21]. This is not the case for canine patients which can present with disease due to *M. tuberculosis*[22].

Contrary to the common presentation of feline tuberculosis as cutaneous lesions on non-pedigree adult cats that hunt, we recently published our findings that we had become aware of three highly unusual systemic/abdominal cases of tuberculosis caused by *M. bovis* that had occurred in young pedigree cats with no outdoor access in two households in England. Of particular note, both of these households are located well within areas of the country deemed to be low risk for *M. bovis* in cattle and other animal species. However, we noted that all three cats had been fed a commercially available complete raw food diet and that the epidemiological significance of this was unclear at the time. Following the publication of letters in the *Veterinary Record*[23] and *Veterinary Times*[24], we have now identified and investigated a total of five households infected with *M. bovis* involving 13 cats; here we report on all of the households and show that the raw food diet is epidemiologically implicated as a possible source of these infections. Further to these cases, we have investigated a continually growing number of cases diagnosed since our original publication which has allowed us to begin a more extensive epidemiological investigation into this outbreak which will be published separately upon its completion.

4.2 Case details

Each cluster is designated by its regional geographical location which are represented on the map below; the locations given are not exact (county level) in order to comply with client data protection (Figure 4.1). Furthermore, a timetable of the key dates for the outbreak are given in Table 4.1.

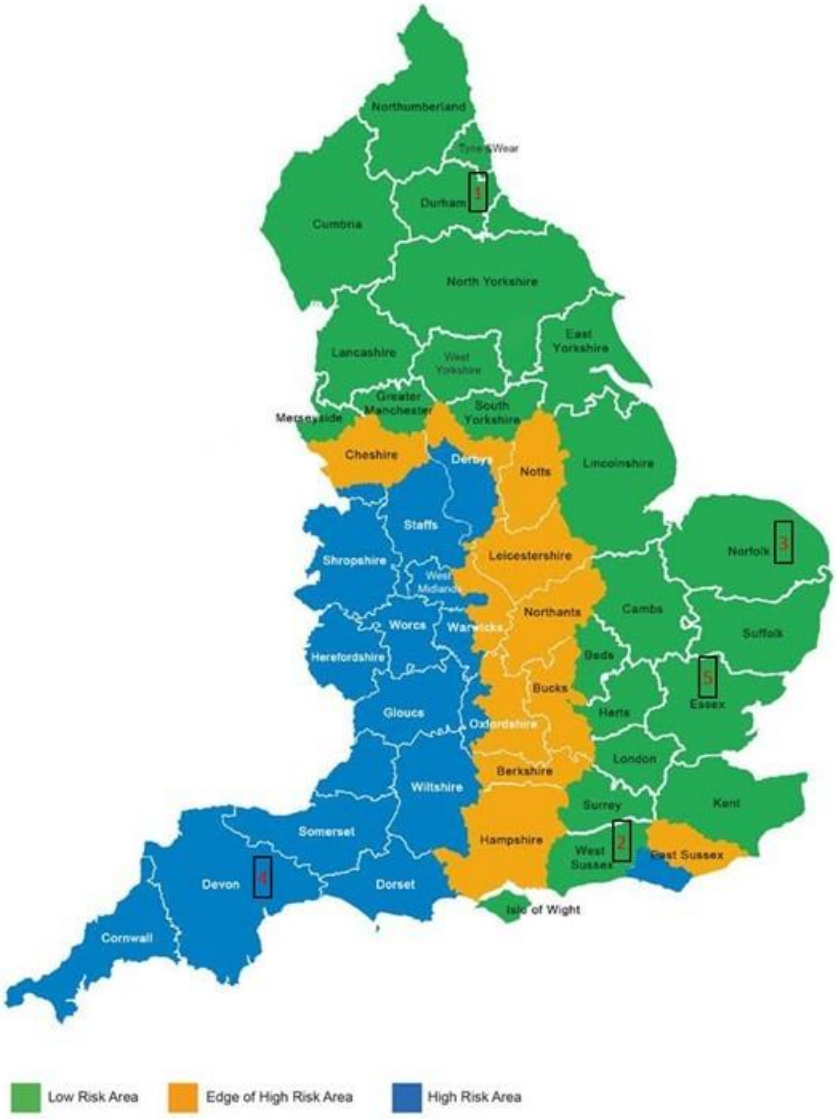

Figure 4.1: A map of the UK showing the approximate location of each of the clusters throughout England. Exact locations are withheld for client data protection. The map is adapted from TBhub (https://www.tbhub.co.uk accessed 3 April 2019), a joint industry initiative, supported by the Agriculture and Horticulture Development Board (AHDB), the Animal & Plant Health Agency (APHA), the British Cattle Veterinary Association (BCVA), the Department for Environment, Food and Rural Affairs (DEFRA), Landex and the National Farmers Union (NFU)

Table 4.1: A timetable of the dates on which the first cat in each cluster was diagnosed with tuberculosis and when the relevant government authorities were alerted to our concerns.

Date (2018)	Event
15th August	First cat in Cluster 1 diagnosed by NR, Wear Referrals
21st September	First cat in Cluster 2 diagnosed by ED, Top Cat Veterinary Centre
24th September	University of Edinburgh (DGM/COH) notify Animal and Plant Health Agency (APHA) and begin a collaborative epidemiological investigation.
29th September	University of Edinburgh (DGM/COH) open investigations with Public Health England
10th October	University of Edinburgh (DGM/COH) contact food manufacturer with concerns
29th October	First cat in Cluster 3 diagnosed by RH, Wymondham Vet Clinic
3rd November	First cat in Cluster 4 diagnosed by JG, City Vets
14th November	First cat in Cluster 5 diagnosed by SVP, Millenium Veterinary Practice
11th December	Food manufacturer recalls suspect product

Cluster 1: Durham

A two year old, male neutered Siamese cat was referred with lethargy, hyporexia and pyrexia (40.5°C) of two weeks' duration. The cat had been acquired from a breeder at 12 weeks of age, had not received any vaccinations since the kitten course, and was indoor-only with one other cat (see below). The cat had been fed only a single commercial frozen raw food diet since acquisition[a]. Routine haematology and serum biochemistry (Table 4.2) documented a mild non-regenerative anaemia. The cat tested negative for FeLV antigen and anti-FIV antibodies. An abdominal mass attributed to an enlarged mesenteric lymph node was palpated and surgically biopsied, with pyogranulomatous, necrotising lymphadenitis identified histopathologically.

Table 4.2: Abnormalities identified on routine haematology and serum biochemistry analysis of the presenting cat in each Cluster of cats.

	Analyte	Measured value	Reference Interval
Cluster 1	Haematocrit	25.8%	27.0-45.0%
	Red blood cell count	$4.7x10^{12}$/L	$5\text{-}10x10^{12}$/L
	Neutrophil count	$12.85x10^{9}$/L	$2.5\text{-}12.8x10^{9}$/L
	Urea	15.4mmol/L	2.8-11mmol/L
	Glucose	8.1mmol/L	4.5-8mmol/L
Cluster 2	Haematocrit	21.8%	27.0-45.0%
	Neutrophil count	$25.00x10^{9}$/L	$1.48\text{-}10.29x10^{9}$/L
	Monocyte count	$2.00x10^{9}$/L	$0.07\text{-}0.85x10^{9}$/L
Cluster 3	Packed cell volume (PCV)	14%	25-45%
	Neutrophil count	$14.34x10^{9}$/L	$1.48\text{-}10.29x10^{9}$/L
	Symmetric dimethylarginine (SDMA)	16µg/dL	Less than 14µg/dL
Cluster 4	Haematocrit	25.2%	30.3-52.3%
	Total white blood cell count	$18.16x10^{9}$/L	$2.87\text{-}17.02x10^{9}$/L
	Neutrophil count	$13.49x10^{9}$/L	$1.48\text{-}10.29x10^{9}$/L
Cluster 5	Haematocrit	21.0%	27.0-45.0%
	Neutrophil count	$24.32x10^{9}$/L	$1.48\text{-}10.29x10^{9}$/L

On presentation at the referral centre, a thin body condition was noted (body condition score 3/9; weight 4.3kg). The respiratory rate was elevated at 42 breaths/min, with wheezing detected on inspiration and expiration. An 8cm firm mass was palpable in the mid-abdomen, without evidence of pain.

Repeated routine haematology and serum biochemistry (Table 4.2) identified marginal mature neutrophilia, mild hyperglycaemia and mildly elevated urea. A computed tomography (CT) scan of the thorax showed a diffuse ground-glass appearance to the lung parenchyma, which also contained disseminated micro-nodular lesions (Figure 4.2). The sternal lymph node was mildly enlarged. A CT scan of the abdomen showed the enlarged and lobulated appearance of the mesenteric lymph nodes (Figure 4.3).

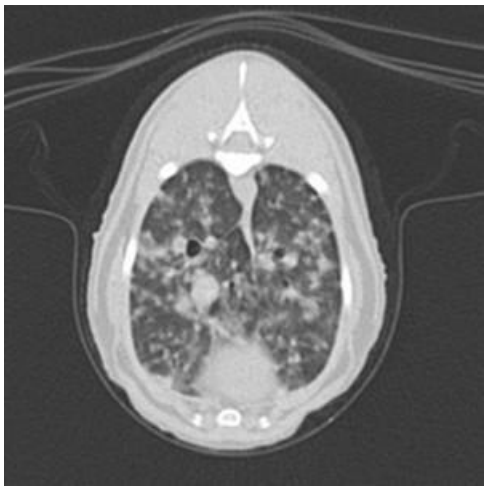

Figure 4.2: Transverse computed tomography (CT) scan of the thorax of the first clinical case from Cluster 1 showing an extensive multifocal nodular and alveolar lung pattern, consistent with mycobacteriosis

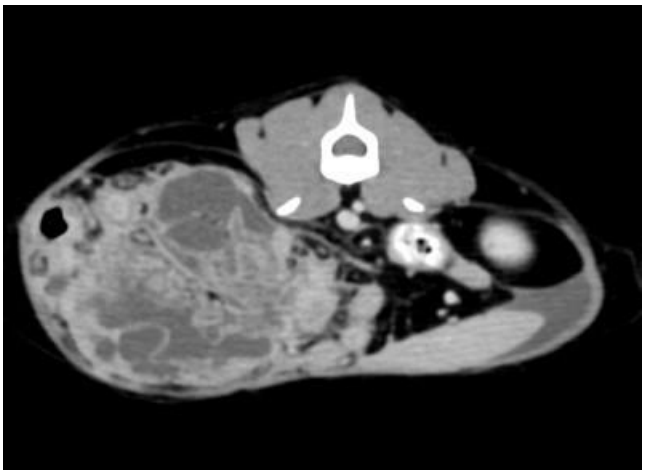

Figure 4.3: A transverse CT scan of the abdomen of the first clinical case in Cluster 1. The scan shows a large, multinodular mass occupying approximately 2/3 of the abdominal cavity.

Lymph node biopsies showed pyogranulomatous lymphadenitis with no infectious organisms seen on Zeihl-Neelson staining. To begin to investigate the differential diagnoses of pyogranulomatous lymphadenitis in the cat, feline coronavirus (FCoV) antibodies, serum

alpha-1-acid glycoprotein and serum albumin:globulin ratio were tested; they were not suggestive of feline infectious peritonitis (FIP), *Toxoplasma gondii* serology was negative, and *Bartonella* species serology was weakly positive (6 [RI <5.5]). On suspicion of mycobacterial infection, an IGRA was conducted at Biobest Laboratories and indicated infection with *M. bovis.*

The cat is currently being treated with the recommended anti-mycobacterial triple antibiotic therapy[1,2], comprising pradofloxacin (Veraflox™, Bayer, 3mg/kg every [q] 24 hours by mouth [PO]), azithromycin (Zithromax™, Pfizer, 9mg/kg q 24 hours PO) and rifampicin (generic, 11mg/kg q 24 hours PO). Two months after starting medication the abdominal mass was no longer palpable and the cat's weight had increased to 5.12kg. Treatment is on-going at the time of writing.

A two-year-old female neutered Oriental cat that resided in the same household as the case described above was referred to the same centre with a chronic intermittent cough, and a three week history of increased respiratory rate and effort, lethargy and hyporexia. The cat was acquired from a different breeder at the same time as the above cat, had not received any vaccinations since the kitten course, and was indoor-housed only. Both cats were fed exclusively on the same commercial complete raw diet[a]. Prior to referral, pyrexia (39.8°C) had been recorded and routine serum biochemistry had identified a significant hyperbilirubinaemia (64 µmol/L; RI 0-12 µmol/L), whilst haematology was unremarkable.

On presentation at the referral centre, the cat's respiratory rate was 100 breaths/min, with bilateral moist rales on thoracic auscultation and a tracheal wheeze on auscultation of the cervical neck. A thoracic CT scan showed a marked diffuse nodular lung pattern with areas of alveolar infiltrate and an IGRA test confirmed infection with *M. bovis.*

This cat was not amenable to medication for behavioural reasons, and her evident respiratory signs increased the risk of transmission to the owners, so she was euthanased. No *post-mortem* examination was conducted.

Cluster 2: Sussex

A 12-month-old female neutered, indoor only domestic short haired cat was presented for a history of lethargy, hyporexia and significant weight loss (reduced by 12.5% over four months, (body condition score 2/5). This cat, and three other co-habiting cats, were fed on the same commercial raw food[a] as those in Cluster 1 since acquisition as kittens. On presentation this cat was pyrexic (41.1°C) and an abdominal mass was palpable cranial to the bladder. The cat was referred for further investigation which revealed mild generalised peripheral lymphadenopathy and diffuse bilateral wheezes and crackles on lung auscultation. Routine haematology and serum biochemistry (Table 4.2) showed non-regenerative anaemia, a mature neutrophilia and monocytosis. Serum biochemistry revealed elevated globulin levels. As for the above Cluster the differential diagnoses included FIP, a lymphoproliferative disease, mycobacterial disease or other infectious agents. The cat tested negative for FeLV antigen and anti-FIV antibodies. A chest and abdominal CT scan revealed multifocal pulmonary nodular-like lesions, alveolar foci and partially consolidated left and right cranial lung lobes, generalised severe lymphadenopathy, a multi-lobulated multi-cavitated right cranial abdominal mass around the ileum with perilesional peritonitis and moderate splenomegaly.

A fine needle aspirate from the enlarged nodes revealed extensive pyogranulomatous inflammation, containing numerous AFB with typical mycobacterial morphology (Figure 4.4a and b). Unstained sections were submitted to Leeds University Mycobacterial Reference Laboratory (LUMRL) for mycobacterial PCR which identified MTBC DNA; subsequent speciation using the GenoType MTBC kit (Hain LifeScience GmbH, Nehren, Germany) confirmed infection with *M. bovis.*

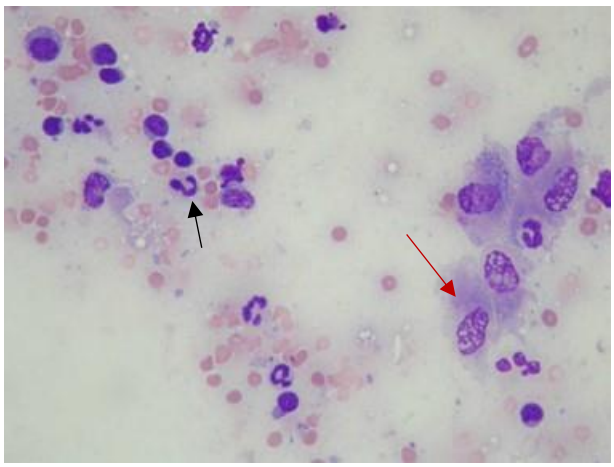

Figure 4.4a: A fine needle aspirate from the inguinal lymph node of the clinically affected cat in Cluster 2. The slide is stained with Wright's stain (40x magnification). It shows reactive, vacuolated "foamy" macrophages (red arrow) and mature neutrophils (black arrow).

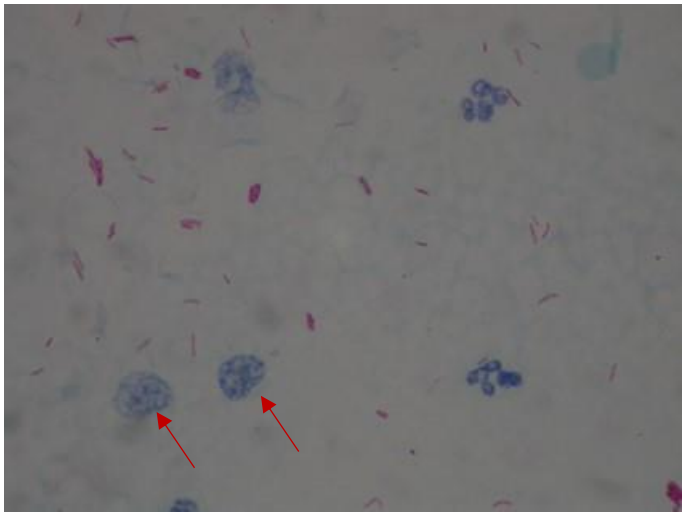

Figure 4.4b: A Ziehl–Neelsen stain of a second slide made from a fine needle aspirate (as shown in Figure 6.3a) viewed under oil emersion (x100 magnification). The slide similarly shows reactive, vacuolated "foamy" macrophages (red arrows). Throughout the slide there are positively stained (fuschia coloured) extracellular rod shaped bacilli with typical mycobacterial morphology.

Given the severity of disease the cat was euthanased on welfare grounds. On IGRA testing, the three co-habiting cats had results indicative of infection with *M. bovis*. They remain clinically well though due to financial restrictions further investigation (*e.g.* diagnostic imaging) has not been undertaken at the time of writing.

Cluster 3: Norfolk

A 15-month-old, male neutered domestic long-haired cat was presented, it had been exclusively indoor housed since acquisition as a kitten along with a female sibling. Both cats were fed exclusively the same brand of raw food[a] as the other Clusters from the time that they were kittens. The cat was presented with diarrhoea and on examination had pale mucus membranes, abdominal distention, poor muscle condition, and marked generalised superficial lymphadenomegaly involving the submandibular, prescapular and popliteal lymph nodes, in addition to an abdominal mass assumed to be enlarged mesenteric lymph node(s). The cat was borderline pyrexic (39.5°C). Routine haematology and serum biochemistry (Table 4.2) revealed anaemia, a mature neutrophilia and a mild increase in SMDA. Fine needle aspirates of the abdominal mass/lymph node showed large numbers of macrophages (Figure 4.5) and intracytoplasmic AFB; confirmatory mycobacterial PCR and speciation at LUMRL on the remaining slide, confirmed MTBC DNA was present but as the slide had been previously stained the DNA was of insufficient quality to differentiate between MTBC organisms *i.e. M. bovis* or *M. microti*. An IGRA test showed a weakly positive result suggestive of *M. bovis* infection. Treatment was attempted with pradofloxacin (Veraflox™, Bayer, 3mg/kg q 24 hours by mouth PO), azithromycin (Zithromax™, Pfizer, 9mg/kg q 24 hours PO) and doxycycline (generic, Summit, 10mg/kg PO q 24 hours). Unfortunately, the cat continued to deteriorate and was euthanased on welfare grounds. The sibling remains clinically well at the time of writing and screening by IGRA test gave a positive result for infection with a non-MTBC mycobacterium species.

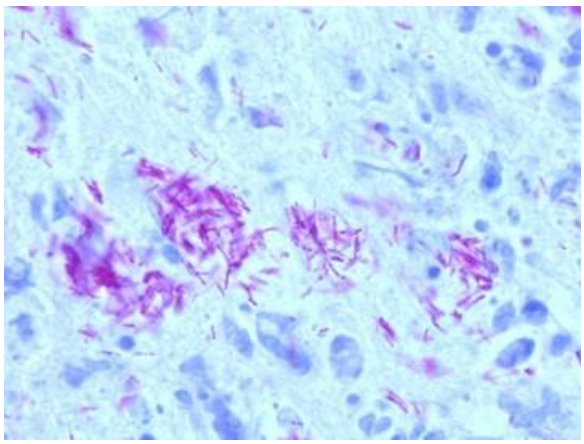

Figure 4.5: A Ziehl–Neelsen stain of a slide made from a fine needle aspirate of the abdominal mass from the case in Cluster 3 viewed under oil emersion (x100 magnification) containing high numbers of AFB.

Cluster 4: Devon

A six-year-old female neutered Bengal-cross cat was presented to its primary veterinary surgeon for lethargy and hyporexia. The cat was an indoor-only cat and had been in the owner's possession since it was seven weeks old. This cat lived with another exclusively indoor-housed cat, a four-year-old male neutered Abyssinian cat that had asthma; both were fed the same commercial raw food diet[a] as the other Clusters since they has been acquired, although they were also given occasional meals of commercial cooked processed fish-based foods[b,c]. On presentation, the cat was pyrexic (39.9°C) with an enlarged inguinal lymph node and localised overlying cellulitis. Routine haematology (Table 4.2) revealed non-regenerative anaemia and leukocytosis due to a mature neutrophilia. The cat tested negative for FeLV antigen and anti-FIV antibodies. Histopathology of the lesion biopsy revealed marked necrotising panniculitis containing AFB with mycobacterial morphology. Unstained sections were submitted to LUMRL for mycobacterial PCR and speciation as described above, and confirmed *M. bovis.* Pending these results, the necrotic area of the lesion was surgically debrided and the cat was given potentiated-amoxicillin (Clavaseptin™, Vetoquinol at 18mg/kg q12 hours PO) and marbofloxacin (Marbocare™, Animalcare at 2mg/kg q24 hours PO). Unfortunately, the wound broke down irreparably; the cat was euthanased on welfare grounds.

A *post-mortem* examination was conducted within the Containment Level 3 facility of the Roslin Institute, University of Edinburgh; there was a surgical defect in the right inguinal area where the affected skin, fat and lymph node had been removed as well as pathological tissue break down (Figure 4.6a and b). On examination of the abdomen many small white, well demarcated hard lesions were present throughout the spleen on both the visceral and cut surfaces. Within the thorax, similar lesions were visible in the caudal lung lobes and pericardium, there was a moderate degree of pericardial effusion.

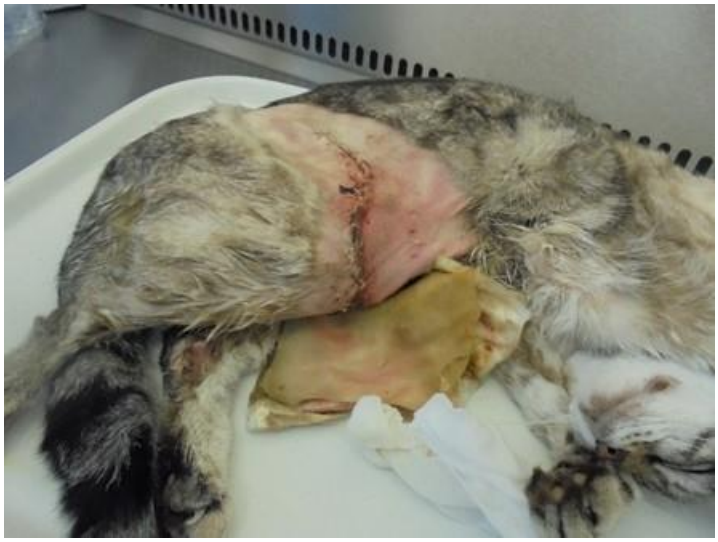

Figure 4.6a: *Post-mortem* examination of a six-year-old female neutered Bengal-cross cat (Cluster 4) shows evidence of the extensive nature of surgical resection required to remove the granulomatous panniculitis present.

Figure 4.6b: Evidence of the breakdown of the surgical wound and surrounding tissue damage which ultimately led to the euthanasia of this patient on welfare grounds.

Grossly pathological tissues were taken from the lung and spleen, homogenised in 1% collagenase solution containing 10% penicillin-G, decontaminated with 4% NaOH for 30 minutes and sown onto OADC supplemented 7H11 (Middlebrook, UK) slopes, Löwenstein–Jensen medium with pyruvate and Stonebrink slopes in order to isolate and genotype the causative strain. After nine weeks, small colonies were visible on the plates with typical morphological appearance of *M. bovis.* In-house PCR testing at Edinburgh followed by PCR performed at LUMRL confirmed the colonies to be *M bovis*; the APHA was notified and sent a second tissue sample for independent culture analysis, which is pending. The in-contact cat remains clinically well at the time of writing but the results of a screening IGRA test indicate *M. bovis* infection. Thoracic radiographs and abdominal ultrasound have identified no pathology. However, this cat has experienced significant recent weight loss (~12% bodyweight), so the owner has elected to treat with standard anti-mycobacterial therapy (drugs as dosages as above in Cluster 1).

Cluster 5: Essex

A 15-month-old, indoor-only, male neutered Maine Coon cat was presented for lethargy and constipation of approximately 48 hours. The cat had been fed exclusively on the same diet[a] as the other Clusters since it was acquired at 12-weeks-old. Physical examination revealed pyrexia (40.0°C) and a palpable, mobile, non-painful abdominal mass in the region of the mesentery. Ultrasound examination confirmed the presence of a 9x13cm mass adjacent to an enlarged mesenteric lymph node and a small amount of free fluid. Fine needle aspirates of the mass revealed large, foamy macrophages, mesenchymal cells and histiocytic infiltration. Routine haematology and serum biochemistry (Table 4.2) revealed non-regenerative anaemia and marked mature neutrophilia.

An exploratory laparotomy was performed and the fluid, mass and mesenteric lymph node were resected (Figure 4.7) and submitted for histological examination. Large numbers of macrophages were observed and a presumptive diagnosis of FIP was made. However, further investigation to confirm FIP, including immunohistochemistry on the biopsy material, reverse transcription quantitative PCR (RT-qPCR) for mutated FCoV on the abdominal fluid, and

serum FCoV titre quantification and alpha-1 acid glycoprotein was not compatible with FIP. During this investigation, retrospective staining of the original biopsy revealed individual AFB with mycobacterial morphology scattered in large areas of necrosis. The cat improved clinically post-surgery but six weeks later developed a cough; thoracic radiography revealed a mass dorsal to the carina (Figure 4.8), whilst a repeat abdominal ultrasound revealed a 2cm mid-ventral mass caudal to the spleen. Cytology of the abdominal mass again revealed a predominance of macrophage infiltration with intracellular non-staining rods present. Cytology with ZN-staining revealed AFB, initial PCR testing at the University of Edinburgh indicated infection with *M. bovis* (protocol as outlined in Chapter 2) and the remaining slides were submitted for mycobacterial PCR and speciation at LUMRL but it was not possible to isolate sufficient mycobacterial DNA for definitive speciation. The IGRA screening tests on the two co-habiting domestic short haired cats which ate mainly commercial dry food, separately from the Maine Coon cat, showed positive responses indicative of *M. bovis* infection though they were clinically well.

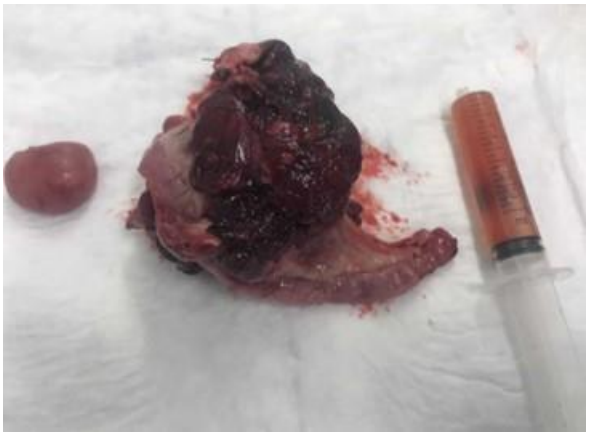

Figure 4.7: The Maine Coon in Cluster 5 had a large abdominal mass (centre) measuring 8x13cm removed during an exploratory laparotomy. The local draining mesenteric lymph node (left) was also removed during surgery as was the ~12ml of ascitic fluid (right). The fluid was tested for mutated feline coronavirus by RT-qPCR but was negative. Retrospective histopathological examination of the mass (centre) showed scattered mycobacteria

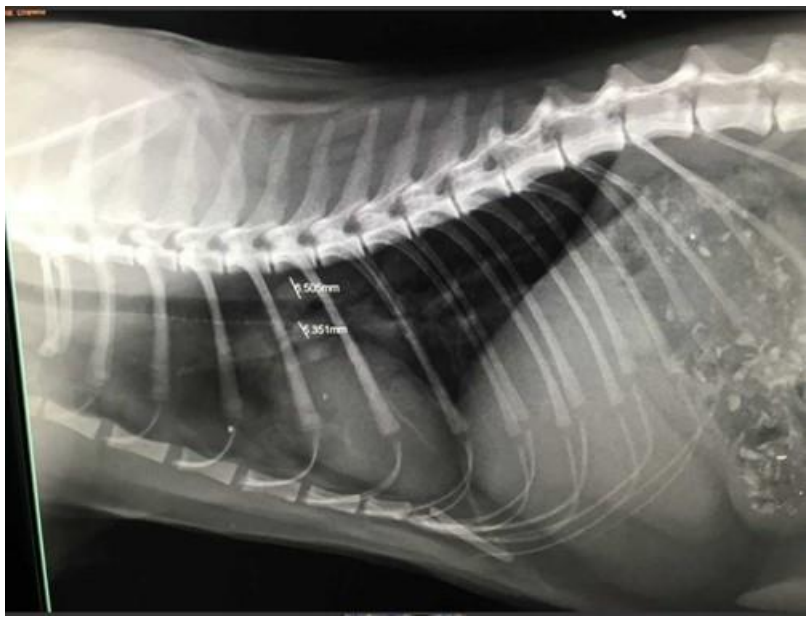

Figure 4.8: A left latero-lateral of the thorax of the Maine Coon cat from Cluster 5. There is a mass (total diameter 1.2cm) at the level of the carina. A cough had become clinically evident at the time this radiograph was taken. It resolved within three days of anti-mycobacterial triple antibiotic therapy.

The Maine Coon was treated with the recommended anti-mycobacterial triple antibiotic therapy[1,2] comprising marbofloxacin (Marbocyl™, Vetquinol, 2mg/kg every [q] 24 hours PO), azithromycin (Zithromax™, Pfizer, 9mg/kg q 24 hours PO) and rifampicin (generic, 11mg/kg q 24 hours PO). Two months after starting medication the abdominal mass was no longer palpable and the cat's weight had increased. However, following owner-led cessation of therapy at this stage, the cat subsequently relapsed and was euthanased on welfare grounds and has been submitted to APHA officials for *post-mortem* examination, the results of which are pending at the time of writing. Due to the owner being immunocompromised, the two in-contact IGRA positive cats were also euthanased.

4.3 Follow-up investigations

The current legislation applicable to England (The Tuberculosis (England) Order 2014) Article 6[25] states that suspicion of tuberculosis in the carcass of any pet mammal is notifiable, and as such the Secretary of State has been duly notified of all of the cases presented here via the APHA. However, there is no statutory obligation of investigation in non-bovine animals and so current investigations have been led by the University of Edinburgh and largely financed, where conducted, by the University and/or owners.

Public Health England (PHE) have similarly been informed of the cases and all owners have been offered the opportunity to discuss the zoonotic aspects that these cases present should they wish to do so with PHE. PHE are currently adopting a precautionary public health approach similar to that which has been described previously[26]. The owners of two of the affected cats were found to be IGRA and Mantoux test (tuberculin skin test) positive respectively, one has required the instigation of anti-tuberculosis medical therapy.

Potential sources of exposure to *M. bovis* were explored with the owners; all except the raw commercial food were excluded. Excluded sources included wildlife contact (all cats were solely indoor cats), the presence of rodent populations inside the buildings in which the cats lived, access to raw milk, and exposure to any known infectious humans.

As the only common factors between all clusters are that they were diagnosed with the same infection (*M. bovis*), were exclusively indoor cats and were fed a commercially available raw food product produced by a single manufacturer[a], the Food Standards Agency (FSA) have similarly been informed and they are investigating appropriately.

The authors at the University of Edinburgh have been sent detailed batch information and photographs of and/or actual samples of the diet from three of the five clusters, as was being fed to the cats at the time the diagnoses were made (Figure 4.9a-c). Notably, all three of these samples were the venison product produced by the same company. On questioning the owners; the venison product from the single manufacturer comprised ~80% of the ration of

one cat (Cluster 4), the sole intake of another (Cluster 5) and the “vast majority” of the diet of a third (Cluster 2). The remaining two clusters (Cluster 1 and 3) fed a variety of the products from the same company, including the venison product.

As such, the evidence we have collected during our investigations of these cases provide compelling, if only circumstantial, evidence of an association between the diet of these cats and their *M. bovis* infections.

The company was alerted, and an internal investigation by them led to the voluntary withdrawal of the venison version of their food from sale; ‘because some of the ingredients were not inspected in line with EU requirements. The absence of inspection means the safety of the product cannot be confirmed and may therefore carry a potential risk.’ The batches withdrawn are those dated as best before between March 2019 and August 2019 and pre-date those submitted for testing at the University of Edinburgh. The FSA are now undertaking this area of the outbreak investigation independently.

Figure 4.9: Three of the five Clusters had retained the food product that the affected cats were being fed at the time that they became ill. One owner sent photos taken by themselves **(4.9a - left)**, the other two owners sent the food to the University of Edinburgh by refrigerated courier and the photos were taken inside the Containment Level 3 facility prior to further testing **(4.9b – centre** and **4.9C - right**).

4.4 Discussion

This outbreak currently includes 13 cats, in five separate Clusters, that were found to be infected with *M. bovis*; six cats were clinically unwell at diagnosis (five of these are now dead), while seven were found to be positive by IGRA *i.e.* they had evidence that they have been infected with *M. bovis,* although how many of these latter cats' are likely to become ill is unclear. Three have not been assessed further by their veterinarian (Cluster 2), and one (Cluster 4) has already lost a great deal of weight, potentially related to *M. bovis* infection (this cat has been treated for *M. bovis* infection and has since recovered all its previously lost weight). While still circumstantial, the only probable source of the infection is that *M. bovis* infected the cats when they were all exposed to the same commercially available raw food. New cases are still occurring and further data collection is in progress at the time of writing, but the authors intend to make readers aware of the details and the scale of this problem as soon as practically possible. The cases reported here appear to be limited to cats which have been fed this feline-specific food (*i.e.* no dogs have yet been affected) and no owners are self-reporting as unwell.

The feeding of commercial raw meat based diets (RMBD) to companion animals has increased substantially in popularity in recent years, not just in the UK but globally[27,28]. In the USA, sales of RMBD doubled in the five years to October 2017 and have expanded by 15.9% in last year alone, making the industry worth an estimated US$195 to the United States economy[29]. In Europe, a recently published study from the Netherlands found that 51% of dog owners fed their animals either completely or partially on RMBD[30]. A similar increase has been seen in the UK, with what were only a handful of companies registered as producers of RMBD a few years ago having increased to a current excess of 80 registered with the Department for the Environment, Farming and Rural Affairs (DEFRA)[27]. Shapiro *et al* (2017)[31] found in a survey of Australian cat breeders that raw meat was fed as an integral constituent of the diet by 89% of respondents. The reasons behind so many people adopting RMBD feeding are numerous and complex. Some owners feel that it is physiologically more "natural" for their pet to consume food similar to that they would have eaten at an earlier stage in their evolution (*i.e.* pre-

domestication)[31-33]. There have been speculative suggestions that RMBD feeding may be beneficial in preventing the onset of dental disease, food responsive inflammatory bowel disease and atopy, and a recent paper has shown that the microbiome of dogs fed RMBD is more diverse than when fed processed diets[31-34]. The topic of what the veterinary profession should be advising clients with respect to RMBD is highly emotive on both sides of the debate, and the evidence for and against is frequently either low quality or simply anecdotal reports with a lack of rigorous, properly controlled trials[31-35]. There is however, consensus that home-prepared RMBD should be generally avoided as it is extremely difficult to make them nutritionally balanced[35]. This concern has been overcome by the introduction of commercially available complete RMBD which comply with the strict legislative requirements, overseen by the European Pet Food Industry Federation (FEDIAF), to ensure nutritional and energy requirements are met if any given product was provided as the sole ration for the life time of the animal in question[36-38].

Unfortunately, statutory regulations governing RMBD only account for the presence or absence of a limited number of infectious organisms[39]. It is one of the few areas regarding RMBD on which there is agreement; that there is a significant increase in the risk of infectious diseases to both pets and owners through the consumption and handling of such products on a regular basis[40-46]. Almost all RMBD are sold frozen in order to extend their expiry dates and, in the case of some organisms (*e.g.* nematodes such as *Trichinella* species) reduce or eliminate contamination[47]. In studies of the zoonotic infectious agents present within thawed RMBD, *Salmonella* species have received the most attention[44-46]. Although subclinical infections occur frequently in animals, *Salmonella* species can cause gastroenteritis and even septicaemia[48-50]. For example, Stiver *et al.* reported fatal septicaemic salmonellosis after RMBD feeding in two cats[51]. Other studies have successfully recovered a variety of viable zoonotic pathogens including *Escherichia (E.) coli* serotype O157:H7, *Listeria monocytogenes*, *Sarcocystis* spp. *Campylobacter* spp. and *Toxoplasma gondii* from RMBD[44-46,52]. At least one such RMBD contaminant, Shiga Toxin releasing *E. coli* (STEC) has been linked to human deaths in the UK, and Public Health England are attempting to educate owners about the risks associated with RMBD[53]. In the USA, raw cat food has similarly had to

be recalled due to contamination with *Listeria monocytogenes*[54]. At this stage, as cases are continuing to be diagnosed there is insufficient evidence to ascertain if this is a single, one off event or an ongoing problem. However, in either instance this outbreak raises concerns over the strength of current regulation with regards to meat inspection procedures and may necessitate an increase in trained veterinary meat inspection of a carcass before it is passed as fit for animal consumption. With particular reference to wild venison, one such alteration could be that the entire gralloch (all viscera) be brought to the slaughter-house for veterinary inspection.

Our study is the first to report infection of companion animals with an MTBC pathogen which appears to be associated with feeding a RMBD, with 13 cats in five households infected, as identified to date. Not only does this imply that the owners were handling contaminated material, but the gastrointestinal presentation means that the cats may have been shedding *M. bovis* into their home environment, creating the potential for even greater exposure to owners. Of further concern, the Pet Food Manufacturers Association guidance to owners handling RMBD is that they should clean surfaces, utensils and animal food bowls with “soap and hot water”[55]. Pasteurisation requires milk to be heated to 71.5°C and held at that temperature for a minimum of 15 seconds to inactivate *M. bovis*[56]; it is highly unlikely that owners would be able to achieve this in a domestic setting, so *M. bovis* is very likely to be able to persist on kitchen surfaces even after the owners feel that they have been “cleaned”. Furthermore, *M. bovis* is able to survive for at least 60 days in standing water[57], such as might be found in or around a kitchen sink, over which time further organisms may be added with each prepared meal making it highly plausible that the small dose needed to infect a human could be reached.

The severity of clinical disease that was seen in the cats we report here is much greater than we would typically expect to see through infections acquired through hunting infected rodents. The clinical signs of pyrexia, anaemia and leukocytosis (caused by a mature neutrophilia) are not typically present in feline tuberculosis cases and the young age of many of the cats affected is also striking, as is that the cats were indoor only, and not from areas of England known to

have endemic *M. bovis* in rodents, badgers, other wildlife and cattle[58-60]. These factors may mislead clinicians presented with future cases to not consider tuberculosis as a potential differential diagnosis, delaying the time until correct diagnosis and thus exposing owners to possible infection for extended periods.

Many of the cats presented in these clusters were either too sick to attempt treatment, or clinically deteriorated despite attempted therapies giving a case fatality rate, to date, of 83% compared to our more usual 70-80% successful treatment rate[2]. The severity of infection was similar to that which we previously reported in an outbreak of *M. bovis* tuberculosis in a pack of working Foxhounds where it was concluded that the source of infection was most likely the feeding of contaminated raw meat[61]. That outbreak resulted in a higher than typical clinical attack rate for tuberculosis (approximately 10% of the group) and when the hounds started to display clinical signs, they deteriorated so rapidly that euthanasia was required on welfare grounds within hours to days[61]. Additionally, feline abdominal *M. bovis* infections acquired in a nosocomial setting and cats with disseminated *M. bovis* infection that appeared to have followed extensive ingestion, also displayed rapid clinical progression, and severe signs such that euthanasia on welfare grounds was quickly required[62,63]. The parallels between these fulminant outbreaks suggests that there may be an underlying difference in the immunopathogenesis of the disease, whereby gastrointestinal/peritoneal challenge of companion animals with *M. bovis* results in more severe disease that is less amenable to treatment and may thus have implications for the clinical management of gastrointestinal tuberculosis cases in comparison to the more typical dermal presentation. Alternatively, or additionally, it may be that strains of *M. bovis* which are capable of establishing infections in companion animals following gastrointestinal/peritoneal challenge may have or may utilise additional virulence factors which make these infections clinically more aggressive. The latter scenario would have profound implications on the zoonotic risk to owners if they were being exposed to *M. bovis* with inherently greater virulence.

The IGRA was used to detect *M. bovis* infection in all six of the unwell and a further six of the in-contact cats. However, one in-contact cat (Cluster 3) was IGRA tested and found to have a

greater response to PPDA than PPDB, *i.e.* a pattern not consistent with that expected of *M. bovis* infection. It may be that this cat has not been challenged with *M. bovis* but has co-incidentally and/or previously been sensitised to antigens of the *M.avium-intracellulare*-complex. However, studies in calves sensitised to environmental mycobacteria and infected with *M. bovis*, have concluded that *M. bovis* infection may be concealed for some time in this situation[64]. It is therefore possible that this cat may develop a different response, *i.e.* that typical of *M. bovis,* if it were to be retested in the future.

Work by our team at the University of Edinburgh is ongoing to develop a rapid PCR assay which we hope can be performed on tissue lysates, (unstained) cytology slides and formalin-fixed biopsy tissues. Where this was used in these cases it proved extremely useful; but, its use outwith a research capacity will require more extensive evaluation before this is made available to clinicians. Similarly, the team is continuing to work with Biobest Laboratories to validate to use of the feline IGRA in the detection and monitoring of tuberculosis in cats.

Urgent work is now needed to establish the extent of any contamination of the RMBD pet food chain in the UK, and a full epidemiological investigation is underway to establish where lessons can be learned in order to future safeguard animals being fed RMBD.

Management of suspect cases

The team at the University of Edinburgh are always keen to hear from clinicians who may have, or have had, suspicions about similar cases and can offer our experience in both diagnosing and managing these patients. Currently, we are advising that animals known to have been exposed to the recalled food, or who have been exposed to a confirmed case but are displaying no clinical signs should be tested by IGRA at least four weeks after the last known exposure. If these tests are positive then diagnostic imaging should be undertaken to assess for structural (*i.e.* active) disease (full body CT or radiographs and, ideally, abdominal ultrasonography). Where present, active disease should be treated appropriately; as outlined in O'Halloran and Gunn-Moore, 2017[1]. There is currently no evidence on which to base the appropriate course of action for animals that are IGRA test positive but lack evidence of

structural disease. It may be appropriate in these cases to closely monitor body weight, condition score and resting respiratory rate and to investigate further if these decline. Alternatively some owners may elect to give prophylactic therapy; isoniazid (which is occasionally used in humans for this purpose) can be used for six months but experience of its use in cats is limited and toxicities may occur. Instead, some owners may choose to treat using the triple combination of anti-tuberculous therapy for three months, given that the use of this protocol is well established and potential side effects are well documented.

4.5 Conclusion

This report follows our initial alert regarding an ongoing outbreak of M. bovis tuberculosis in pet cats across the UK. It describes six clinically sick cats and seven in-contacts with *a* mortality rate of 83%. Lifestyle investigation revealed the common factors between clusters were that the cats were exclusively indoor only and fed a commercially available raw food product produced by a single manufacturer. The Food Standards Agency, Animal and Plant Health Agency, Public Health England and the food manufacturer concerned have been notified/informed. Our results provide compelling, if circumstantial, evidence of an association between the commercial raw diet of these cats and their *M. bovis* infections.

4.6 References

[a]Natural Instinct®; https://www.naturalinstinct.com

[b]Sainsbury's Delicious Recipes 1+ Adult Cat Food Tuna in Jelly

[c]WHISKAS® 1+ Years Complete Dry Cat Food with Tuna 340g

[d]https://www.tbhub.co.uk/ Accessed 3rd April 2019

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PART 3

Tuberculosis in UK cats associated with a commercial raw food diet.

Editorial, *Journal of Feline Medicine and Surgery,* August 2019

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Johann Schonlein coined the term "tuberculosis" in the year 1834, 48 years before the causative agent *Mycobacterium (M.) tuberculosis* was first visualised by Robert Koch. At that time, one in seven of the world's population was thought to be infected and despite significant efforts in the intervening almost two hundred years, this prevalence has actually increased to an estimated one in three. Whilst *M. tuberculosis* may have been the first tuberculous organism identified, it is restricted to infecting a very small number of species. By comparison, the causative agent of 'zoonotic tuberculosis', *i.e. M. bovis,* has a very wide host range. So named for is predilection for bovine hosts, *M. bovis* can actively infect an exceptionally broad range of species which include but are not limited to; cattle, badgers, deer, camelids, a variety of rodents, human and non-human primates, racoons, possums, and coyotes. Not surprisingly, our feline patients are also susceptible.

Last year, we were alerted to a highly unusual group of feline *M. bovis* tuberculosis cases (O'Halloran *et al.* 2018a). Initially, the striking feature was that these cases presented with a rare manifestation of disease; alimentary tuberculosis (Figure 4.10): this compares to most 'typical' cases of tuberculosis in cats in the UK, which are cutaneous and/or pulmonary. The investigation became even more complex when diagnostic tests confirmed *M. bovis* infections; this was unexpected as these cats were living in areas of the UK which have either a very low

prevalence of *M. bovis* in cattle and badger (such as Lancashire) or are officially *M. bovis* free (such as Scotland).

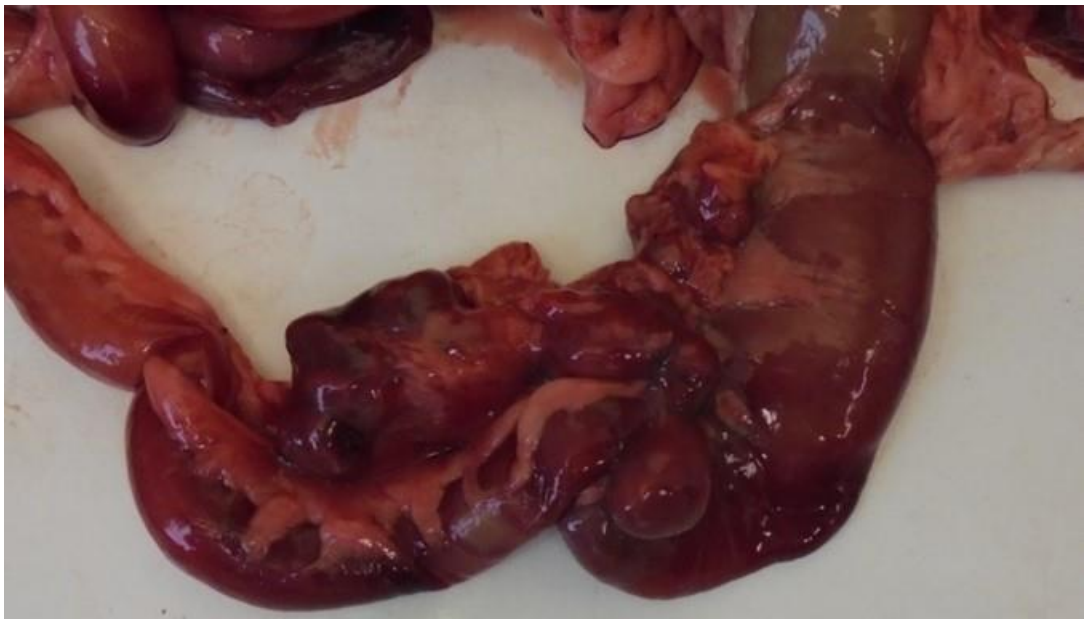

Figure 4.10: The grossly abnormal intestine and associated lymph nodes removed for specialist mycobacterial culture from a one year old, male neutered Persian cat who was weaned onto the Natural Instinct Wild Venison product and continued to eat it as a sole ration until developing marked chronic weight loss, hyporexia and an expiratory wheeze. He was euthanased on welfare grounds due to the severity of clinical signs.

We investigated the lifestyle histories of the cases to try and identify any possible routes of exposure to *M. bovis.* We discovered that all of the cats were indoor-only (*i.e.* they were at negligible risk from wildlife or rodents), they were of different breeds and were mostly young, none had been fed raw (unpasteurised) milk, or lived with people with active tuberculosis. The only common feature was that all were fed (in varying proportions) a commercial raw food diet; Natural Instinct, Wild Venison (Figure 4.11). On discussion with the company and from their subsequent investigations, it was concluded that some of the ingredients had not been meat-inspected in line with EU requirements so the safety of the product could not be confirmed; the company voluntarily recalled the food.

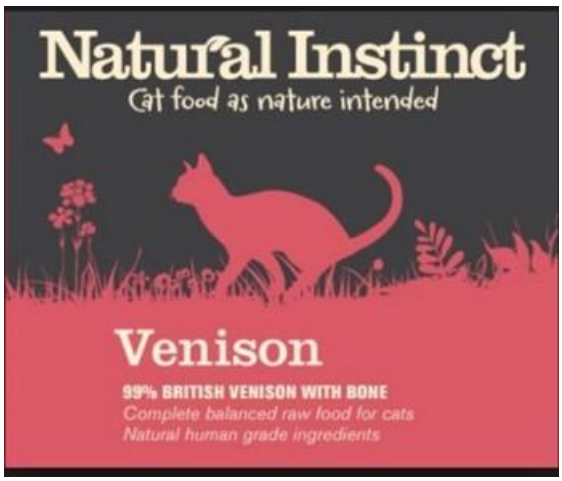

Figure 4.11 The Natural Instinct product linked to this outbreak.

The EU requirements in question (predominately EU Regulation 853/2004) state that a wild venison carcass (including its gralloch, or innards) must be inspected by a trained person (which can be the stalker) after the deer's death to look for any signs of disease, parasites or contamination. However, the stalker does not have to bring the gralloch 'off the hill', so infections could be missed. In addition, the supply by hunters of small quantities of wild game meat directly to local retailers is not covered by these regulations and therefore is not subject to any of the defined rules for food of animal origin.

In this case, possible contamination could have been avoided if this Regulation had been correctly adhered to. However, in order to reduce the risk of a similar incident occurring in the future it is our view that examination of entire game carcasses (including the gralloch) should be undertaken by a qualified, specially trained veterinary surgeon in an abattoir if the meat from the carcass is intended for consumption as a raw food product (for either people or pets). Such a policy change could be applied across England and Wales, or limited to regions within the high risk and edge areas for endemic *M. bovis* infections in cattle and wildlife. Additionally, we feel it would be sensible to prevent the feeding of raw offal to any companion animal. This policy is already in effect for packs of working hounds; since October 2017 the feeding of offal from livestock species to registered kennel hounds has been banned in England following our

previous work investigating another raw-food associated *M. bovis* outbreak and no new cases have since been reported (Anonymous, 2017; O'Halloran *et al.* 2018b).

Our initial investigation into this current outbreak has been reported in this publication previously (O'Halloran *et al.* 2019) and new cases continue to occur. To date we have diagnosed 45 cases with active clinical disease, 73 with inactive (*i.e.* latent) infection and screened in excess of 50 well cats where owners are concerned regarding historical exposure to the contaminated product. A more up to date report of our findings is in preparation and will be published as soon as possible.

Author Contributions:

O'Halloran, C. wrote the manuscript under the supervision of Professor Gunn-Moore.

References:

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PART 4

Feline tuberculosis caused by *Mycobacterium bovis* infection of domestic UK cats associated with feeding a commercial raw food diet.

Acknowledgements

Thanks go to all of the veterinarians and owners of these cats for facilitating the investigation and giving permission for the findings to be published. Additional thanks go to the APHA staff who assisted with co-ordinating the epidemiological investigation and conducting *post mortem* examinations and laboratory testing; Tony Roberts (APHA Wallingford), Jeff Jones (APHA Carmarthen), Livio Pittalis & Cornelia Bidewell (APHA Bury St Edmunds), Carlo Bianco & Pedro Sanchez-Cordon (APHA Weybridge), Harriet Stacey (APHA Starcross), Sheila Corrieri (APHA Perth), Malgorzata (Gosia) Dabrowska-Jones (APHA veterinary adviser for non-bovines), Ricardo de la Rua-Domenech (APHA Veterinary Adviser (policy) to DEFRA TB Programme), Eleftheria Palkopoulou and Vivienne MacKinnon (Veterinary Lead Field Services North Scotland).

Abstract

Mycobacterium (M.) bovis can infect cats and has proven zoonotic risks for owners. We describe an outbreak of *M. bovis* in cats from across England and Scotland associated with feeding a commercial raw food diet. Investigations of (pyo)granulomatous lesions, lymphadenopathy, and pulmonary and/or alimentary disease of 45 affected cases over one year identified infections with *M. bovis* genotype 10:a.

Infection was confirmed by culture in a small number of cases (n=5); PCR was used in combination with or as an alternative to culture (n=12) and/or infection was indicated by positive interferon gamma release assay (IGRA; n=34). At-risk cats were screened by IGRA; follow-up testing identified 77 sub-clinically infected cats.

The five culture-positive cases were distributed across England and Scotland, within areas of the UK considered to be low-risk for endemic *M. bovis*. Whole genome sequence analysis of recovered isolates revealed identical nucleotide sequences providing evidence that these infections are epidemiologically linked.

Lifestyle investigations revealed that affected cats were mainly indoor-only, all of whom had been fed Natural Instinct's Wild Venison food as at least part of their diet. As far as possible, other possible sources of infection were explored and were excluded; including wildlife contact, access to raw milk, the presence of rodent populations inside the buildings in which the cats lived, and exposure to known infectious humans.

Owners and examining vets of test-positive cats were referred to their local health professionals; four owners and one veterinary surgeon were found to have a high likelihood of latent tuberculosis infections. One pregnant owner underwent prophylactic treatment. Though it is not possible to conclusively demonstrate a zoonotic origin for these infections, the potential for this should be considered when possible cases are identified.

Our results provide strong evidence that the commercial raw diet of these cats was the likely route of their *M. bovis* infections.

4.7 Introduction

Mycobacterium (M.) bovis is one of the member species of the *Mycobacterium tuberculosis*-complex (MTBC) which are capable of causing tuberculosis across a broad taxonomy of species including but not limited to humans, cattle, deer, dogs and cats (Sales *et al.* 2001; Philips *et al.* 2003; Allen, 2017).

Feline infections with *M. bovis* are strongly co-localised to areas of the UK with a high prevalence of infections in cattle (Gunn-Moore *et al.* 2011); termed high risk areas (HRA) of the UK by the Department for Environment, Food and Rural Affairs (DEFRA). Conversely, *M. bovis* infections are very rare in low risk areas (LRA) and somewhat higher incidence in the edge zones between LRA and HRA regions as evidenced by a culter of *M. bovis* infections in cats in Berkshire in 2013 (Roberts *et al.* 2013).

Within the MTBC group of pathogens, *M. bovis* stands out as the least host restricted and thus has significant zoonotic potential (Sales *et al.* 2001; Biet *et al.* 2005; Suzuki *et al.* 2010). Disease in humans due to infection with *M. bovis*, termed 'zoonotic tuberculosis' by the World Health Organisation (WHO), is a major global public health priority which resulted in nearly 150,000 cases and at least 12,500 deaths worldwide in 2010 (World Health Organisation, 2017). Clinically, about a third of feline mycobacteriosis cases in the UK are caused by MTBC pathogens; in the only published study, *M. microti* was cultured from 19% of all submitted cases of feline mycobacteriosis, and 15% were caused by *M. bovis* (Gunn-Moore *et al.* 2011a; O'Halloran and Gunn-Moore, 2017).

Characterisation of the typical clinical manifestations of feline mycobacterial diseases in the UK has shown that 74% of cases present with single or multiple cutaneous lesions, and 10-16% with pulmonary or systemic signs (Gunn-Moore *et al.* 2011a). The remainder of cases are small numbers of relatively rare presentations such as alimentary, joint or ocular mycobacteriosis (Gunn-Moore 2014; Lalor *et al.* 2017; Stavinohova, *et al.* 2019).

We have previously reported on a highly unusual group of 13 feline *M. bovis* tuberculosis cases (O'Halloran *et al* 2018a; O'Halloran *et al.* 2019). Initially, the striking feature was that these cases all presented with one of the rarest manifestations of disease, alimentary tuberculosis. The investigation became more complex when diagnostic tests indicated *M. bovis* infections as these cats were living in areas of the UK which have either a very low prevalence of *M. bovis* in cattle and badger (such as the North East of England) or Scotland, which holds officially *M. bovis* free status (for cattle (APHA, 2017; O'Halloran *et al.* 2019). Suspicions were raised with regards to a possible link to diet as all cats were indoor-only, had no access to alternative sources of infection as far as we could determine, and they were all fed a single commercial raw food product, Natural Instinct Wild Venison (Food Standards Agency, 2018; O'Halloran *et al.* 2019).

We concluded that these initial findings provided circumstantial evidence of an association between the commercial raw diet of these cats and their *M. bovis* infections. Since then, further cases have been diagnosed and an epidemiological investigation undertaken; the purpose of this publication is to outline in full our findings and conclusions from both the clinical and epidemiological investigations.

4.8 Clinical Investigations

4.8.1 Clinical Presentation of Cases

Forty-five cats from 41 households have been diagnosed with active tuberculous disease linked to this outbreak at the time of writing. All 45 cases were presented to their primary veterinary surgeons over the course of one year (July 2018 to July 2019). The majority of affected cats (39/45, 86.7%) were pedigree breeds including; eight Bengal cats, seven Persians, seven Maine Coons, six Siamese, four British Shorthairs, two Ragdolls and one each of Burmese, Toyger, British Blue, Sphinx and one Bengal cross cat. The remaining six cats were non-pedigree domestic shorthairs. Cases came from all over England and mainland Scotland.

The age at presentation ranged from three-month-old kittens to 13 years, with a median age of two years. There was no statistical difference between the number of male or female cats presenting with disease (X^2_1=1.48, p>0.05); half of the cats (22/45, 48.8%) were neutered males, ten cats were neutered females (22.5%), whilst nine were entire females (20.0%) and four (8.9%) were entire males.

Clinical signs noted by the owners of these cats were most frequently non-specific signs of ill health, including combinations of lethargy (18/45 cats, 40.0%), hyporexia (15/45 cats, 33.3%) and poor or declining body weight/condition (15/45 cats, 33.3%).

The clinical abnormality most frequently reported by examining vets was “respiratory signs”, which was recorded in 75.6% (34/45) of cases. The severity of these signs ranged from mild increases in respiratory rate and/or effort through to a persistent cough and pneumonia causing life-threatening dyspnoea; it was the recorded reason for euthanasia in two cases (Figures 4.12a and 4.121b). In a further three cases (6.7%) thoracic lymphadenomegaly of the peribronchial, sternal and/or mediastinal lymph nodes was the only gross lesion detected on investigation of the primary presenting complaint (lethargy in two cats and hyporexia in one cat).

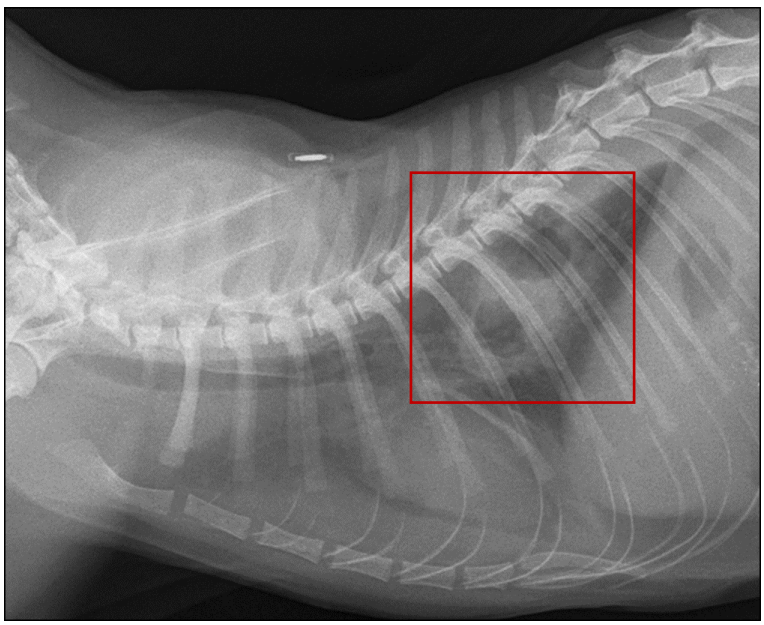

Figure 4.12a: A latero-lateral thoracic radiograph of an affected cat (a four-year-old female neutered indoor-only Bengal cat) showing two cavitating lesions (red box) within the lower respiratory tract. The cat clinically deteriorated and was euthanased on welfare grounds.

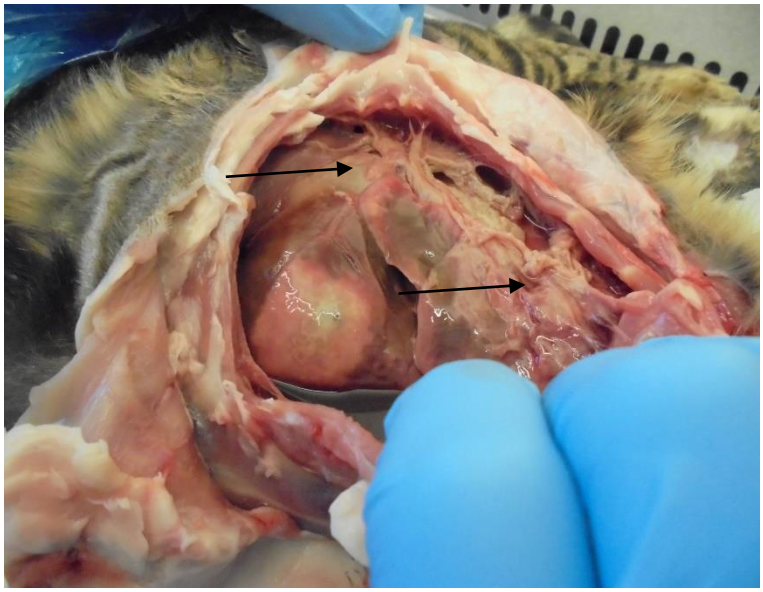

Figure 4.12b: *Post mortem* examination of the same cat radiographed in Fig. 4.12a revealed extensive fibrinopurulent exudate (black arrows) in the thoracic cavity and the extent of the granulomatous lesions. Lung samples submitted for mycobacterial culture were positive for *M. bovis.*

Abdominal masses (granulomas) were palpable in 23 of the 45 cats (51.1%), whilst the abdominal viscera (excluding lymph nodes) were found to be structurally abnormal (either enlarged or containing granulomatous lesions, Figure 4.13) in a further four cases (8.9%) as visualised by abdominal radiography, ultrasonography and/or computed tomography (Figure 4.14). The abdominal lymph nodes associated with the alimentary system (Figure 4.15) were enlarged in four cats (8.9%) and enlarged as part of a generalised lymphadenomegaly in a further eight cats, hence 12 cases (26.7%) had abdominal lymphadenomegaly. Overall, the alimentary tract and associated structures were found to contain gross pathology in 31 of the 45 cases (68.9%), but clinical signs directly relatable to the gastrointestinal system (vomiting, diarrhoea and constipation) where the main presenting sign in only six cats (13.3%).

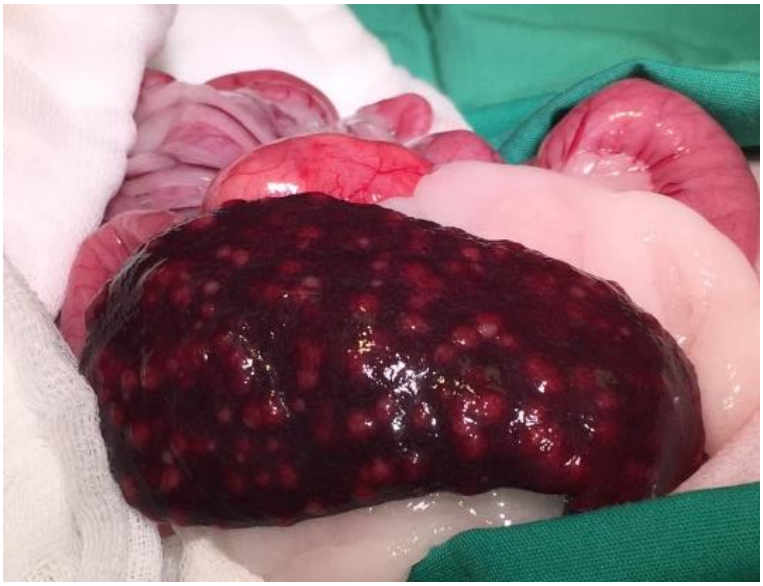

Figure 4.13: The spleen of an 18-month-old male neutered Burmese cat at laparotomy surgery showing multifocal raised white-pink circular lesions later confirmed as granulomatous on histopathology and containing acid-fast bacilli with mycobacterial morphology. The liver of this cat was similarly affected

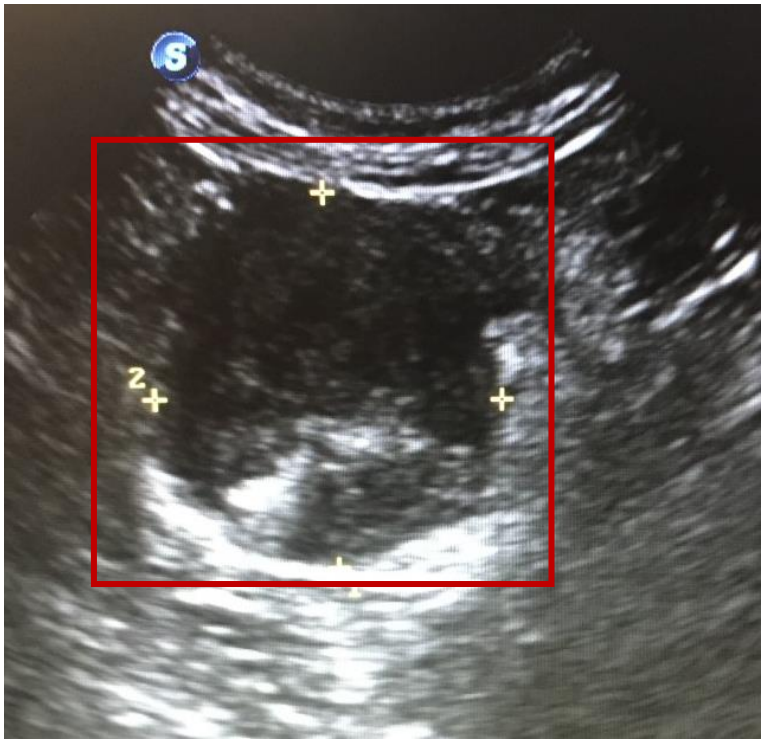

Figure 4.14: An ultrasound examination of an 18-month-old male neutered indoor only Maine Coon cat showing an approximately 2x2.5cm mass (red box) in the abdominal cavity.

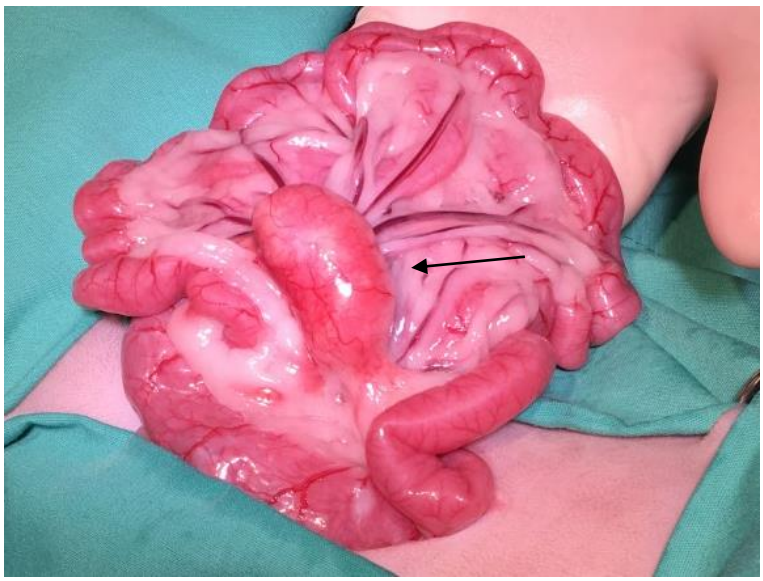

Figure 4.15: A markedly enlarged mesenteric lymph node (black arrow) from the same cat as Figure 4.14 visualised during the laparotomy surgery.

Uncommon clinical signs included non-healing skin lesions (Figure 4.16) in four cats (8.9%), orthopaedic involvement in two cats (4.4%) and optic disease (Figure 4.17) in two cats (4.4%).

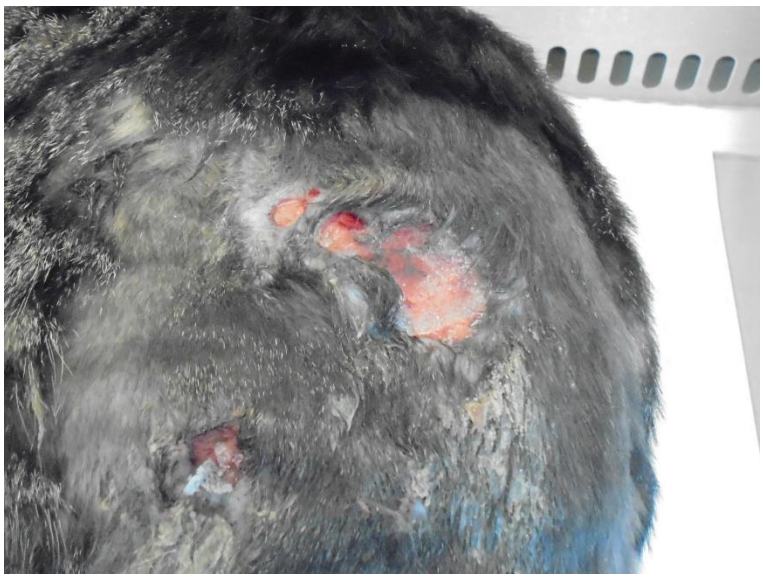

Figure 4.16: The non-healing skin wound over the left pelvis of a two-year-old male, indoor only, neutered domestic shorthair cat that was subsequently culture positive for *M. bovis.*

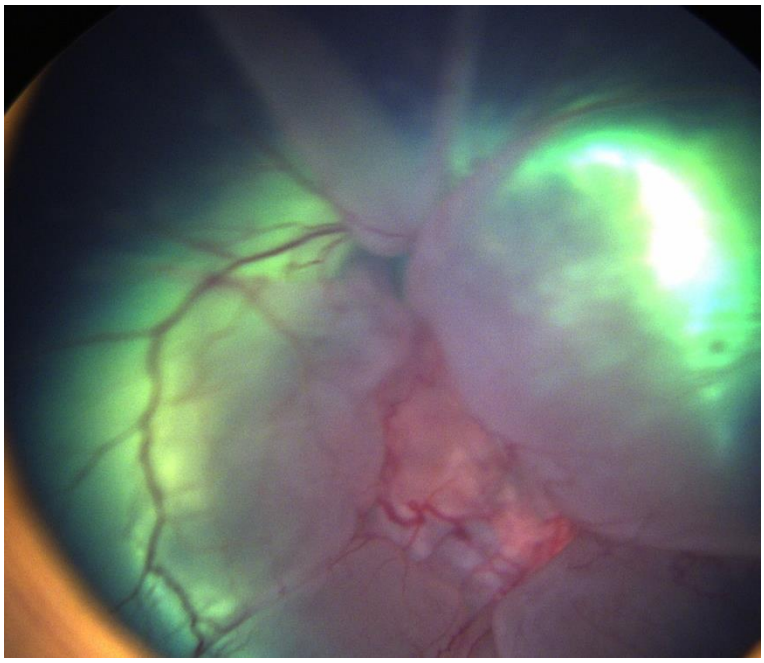

Figure 4.17: Optic examination of a two-year-old indoor only female neutered British Shorthair cat presented for conjunctival hyperaemia revealed a mass over the optic nerve, later diagnosed as granulomatous chorio-retinitis with acid-fast bacilli as well as the observable retinal detachment that was also confirmed histologically following enucleation.

At clinical examination pyrexia was present in 17 cats (37.8%); where recorded, the temperature ranged from 39.8ºC to 41.1ºC, with a median of 40.0ºC (normal range 37.7°C - 39.1°C). Routine haematological evaluation revealed a non-regenerative anaemia in a further ten cats (22.2%) and a mild to moderate mature neutrophilia in six cats (13.3%). Serum biochemistry was largely unremarkable; only two cats (4.4%) showed increased liver enzyme activity, affecting both alanine aminotransferase and alkaline phosphatase. One of these cats had a large hepatic granuloma extending into the biliary duct, whilst the cause of these elevations was not identified in the second cat.

When tested, all cats were found to be negative for Feline Leukaemia Virus antigen and anti-Feline Immunodeficiency Virus antibodies.

4.8.2 Mycobacterial Testing

Specialist mycobacterial culture and subsequent genotyping at a national reference laboratory (Animal and Plant Health Agency [APHA], Weybridge in the UK) remains the only test that can

result in official confirmation of *M. bovis* infection of companion animals (Middlemiss and Clark, 2018). From this outbreak, 11 grossly diseased tissue samples were obtained at *post mortem* examination and submitted for mycobacterial culture at APHA Weybridge. All *post mortem* examinations were conducted either at APHA veterinary investigation centres by APHA pathologists or at the Roslin Institute, University of Edinburgh under Containment Level 3 conditions by the authors (COH/JH). The results of ten cultures are available at the time of writing with the results from one case still pending. Five of these ten cultures (50.0%) resulted in positive results and genotype analysis has identified all five isolates as genotype 10:a. The five cats were resident across the UK in LRA locations. Subsequent whole genome analysis of the cultured isolates revealed them to be identical at the nucleotide level.

Although it is the gold standard test, mycobacterial culture does have a number of limitations. For example, as a prerequisite it requires the acquisition and submission of fresh biopsy samples; however, given the location of many of the lesions in these cases inside body cavities (*i.e.* the chest and abdomen) this made retrieving enough sample for culture *ante mortem* an invasive procedure that many of these cases were not clinically fit to undergo. For most of these cats it was only possible to obtain very small samples by needle aspiration or tracheal/bronchial wash samples for cytological examination (Figure 4.18). In a number of cases where larger biopsies had been obtained, they had been formalin-fixed and submitted for histopathological examination and as such were unavailable for culture. For these cats, we utilised molecular testing methods (GenoTypeMTBC; Hain Lifescience GmbH, Germany) at Leeds University Mycobacterial Reference Laboratory (LUMRL). This methodology is used as standard of care in the diagnosis of human tuberculosis, and although not specifically validated for companion animal tissue samples, this assay is able to diagnose human mycobacterial infections as being caused by MTBC organisms and then specific gene probes are used to determine the infecting species within this group, including the identification of *M. bovis* (National Institute for Health and Care Excellence, 2016). Twelve cat samples of the 15 tested generated positive PCR test results; one indicated infection with an MTBC organism but there was insufficient DNA for speciation and 11 were identified to the species level as *M. bovis*

infections. Two of the *M. bovis* positive test results were subsequently confirmed by mycobacterial culture of lesioned tissue samples.

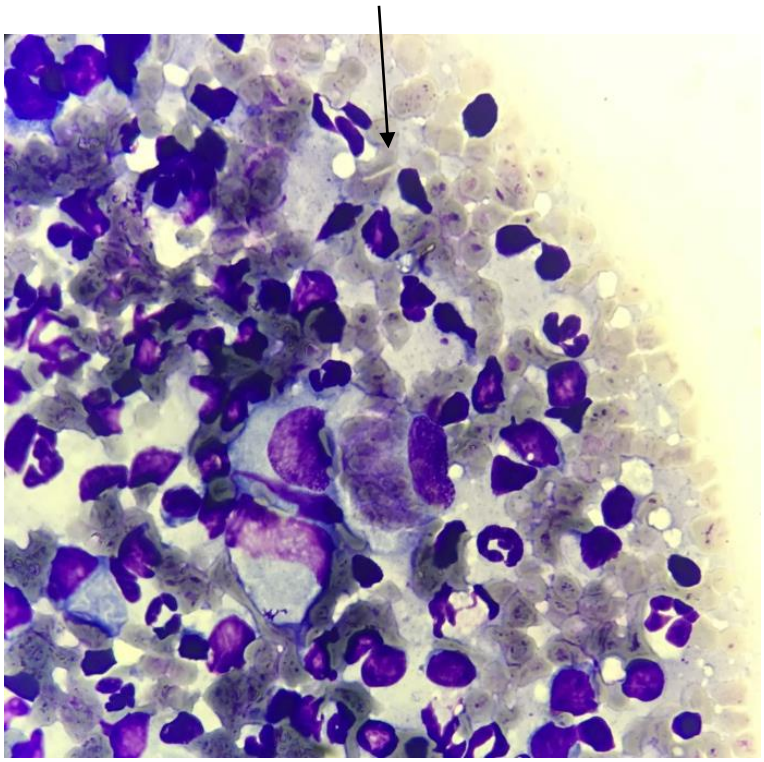

Figure 4.18: A cytological sample obtained by fine needle aspirate from the submandibular lymph node of a two-year-old entire, indoor-only female Bengal cat. The sample is stained with a commercial Romanowsky stain variant (Diff-Quik™) and reveals pyogranulomatous inflammation typical of feline mycobacterial infections with non-staining 'ghost' bacilli typical of *Mycobacteria* spp. (black arrow). Image taken under oil emersion at x100 magnification.

The cat identified by PCR as being infected with an MTBC organism was included in this outbreak investigation even though molecular testing did not identify *M. bovis* DNA. This cat was an 18-month-old indoor-only domestic shorthair which had been fed exclusively on the implicated diet since its acquisition by the owners at eight weeks of age, it had a contemporaneous interferon-gamma release assay (IGRA) result indicative of *M. bovis* infection, as well as lymph node aspirate cytology which confirmed the presence of acid-fast bacilli with mycobacterial morphology. Cumulatively these findings were taken to support its inclusion as an affected cat.

Immunodiagnostic assays for the diagnosis of tuberculosis are used globally to test human and bovine populations (Wood and Jones, 2001; McCormick-Baw *et al.* 2018). The most

successful of these to date is the IGRA; first developed to aid in the eradication of *M. bovis* from cattle populations in Australia, the assay detects antigen-specific T-cell responses to mycobacterial antigens from circulating leukocytes to diagnose infection (Rothel *et al.* 1992; Wood and Jones, 2001). This first test, the BOVIGAM™ assay, is now widely used to detect the cell-mediated immune response to *M. bovis* in cattle, sheep, goats, buffalo and bison (Wood and Jones 2001; Pesciaroli, M. *et al.* 2014, Anusz *et al.* 2017). With small adaptations the test protocol has proved to be extremely useful in humans, and the WHO's new 2018 guidelines on the management of human tuberculosis now support the global use of IGRA testing at-risk populations (Chee *et al.,* 2018, McCormick-Baw *et al.* 2018). In 2008, adaptations to the test procedure were undertaken by the TB Research Group at the then Veterinary Laboratories Agency, now APHA, to optimise the test for domestic cats (Rhodes *et al.* 2008a). The resultant feline IGRA is now commercially available via Biobest Laboratories, Edinburgh, UK and although the number of tested cats remains too small to allow official validation to World Organisation for Animal Health (OIE) standards, several publications have indicated that this is a useful test to generate rapid information on the infection status of cats (Rhodes *et al.* 2008b, Rhodes *et al.* 2011, Gunn-Moore *et al.* 2014). The methodology and interpretation of this test has been previously published and the same protocol has been used for cats involved in this outbreak (Rhodes *et al.* 2008a; O'Halloran *et al.* 2019).

The advantages of IGRA tests compared to other mycobacterial tests are that they can be performed rapidly, only require venipuncture of the patient, rather than more invasive tissue biopsy, and they can support a diagnosis in the absence of an aetiological agent as is common in feline tuberculous granulomas, which are usually paucibacillary (Wood and Jones, 2001; Gunn-Moore *et al.* 2011b). It can be particularly useful for the diagnosis of cases where the lesion is completely inaccessible, such as the two cases of chorio-retinal disease. Of particular relevance to this investigation is that this test is currently the cheapest option available to owners. The combination of lack of invasiveness, speed of generating results, and lower cost are the likely reasons for the high number of IGRA tests in this instance rather than the validated gold standard culture.

During this outbreak 34 of the 45 clinical cases (75.6%) tested positive using the feline IGRA and gave test results indicative of *M. bovis* infection. Of these, eight cases (23.5% of positive IGRA tests) were supported/confirmed by either PCR test (five cats) or subsequent positive cultures (three cats).

4.8.3 Sub-clinical Cases

As the outbreak continued and the commercial raw diet was implicated, owners who fed the diet became concerned that their cats could be infected. In addition, some of the affected cats lived with other cats, so some owners were worried that their other cats could be at risk. Since the IGRA is the only test for mycobacterial infection that can be performed on an animal with no visible lesions many of these owners chose to have their cats tested using this assay. To date, 143 at-risk, clinically well cats have been tested by IGRA, of which 83 (58.0%) results indicate likely *M. bovis* infections.

In a previous outbreak, we utilised an IGRA for screening kennel-housed Foxhounds with a similar test protocol to the feline IGRA (O'Halloran *et al.* 2018b). At that time, we considered that a positive interferon gamma response to mycobacterial proteins/peptides was indicative of a significant antigen specific T-cell response. Furthermore, such a response was thought to be suggestive of significant challenge and probable infection, rather than just exposure to these mycobacteria. We therefore applied the same criteria to these cats and advised that the 83 cats that generated positive IGRA results should be further evaluated for the presence of lesions by full clinical veterinary examination and diagnostic imaging. In six cases (7.2%), imaging revealed mild interstitial pulmonary disease (Figure 7) and associated lymphadenomegaly that were not causing clinical signs, this allowed the clients to treat these cats at an early stage of infection (see below).

The remaining 77 cats were IGRA positive with no detectable clinical disease. These cats were deemed to be likely to be sub-clinically infected and owners were advised that they should be closely monitored for the onset of clinical signs and/or prophylactic therapy could be instigated at their discretion.

4.8.4 Current Approach to Suspect Cases

The team at the University of Edinburgh are always keen to hear from clinicians who may have, or have had, suspicions about similar cases to continue to gather evidence about this outbreak as well as offering our experience in the diagnosis and management of these patients.

Currently, the number of newly diagnosed clinical cases appears to be in decline. The first cases were presented in July 2018 and peaked in June 2019, six months after the implicated product was recalled. No new clinically active cases have been diagnosed, to our knowledge, since the middle of July 2019.

The *ante mortem* testing of any suspect cases can be performed as described earlier – by specialist mycobacterial culture at APHA Weybridge (the gold standard confirmatory test) or by PCR and/or IGRA. Where present, the treatment of active feline tuberculous disease should be carefully considered; there is no requirement to euthanase *M. bovis* infected pets in the UK, but appropriate therapy requires long-courses (typically 3-6 months) of combination antibiotic administration which can be difficult to achieve in cats, can cause side effects and be costly for owners. If treatment is to be attempted then it must be done appropriately, consisting of a combination of a macrolide/azalide, a fluoroquinolone and rifampicin administered daily as outlined in O'Halloran and Gunn-Moore (2017), to prevent the induction of antimicrobial resistance to these medications and to protect public health.

Veterinary clinicians in the UK should be aware that, under the Tuberculosis (England) Order 2014 and equivalent Statutory Instruments in Scotland and Wales (Anon, 2014), in the event that non-bovine animals are euthanased or die when there is suspicion of *M. bovis* infection then this is notifiable to the Secretary of State (via APHA). The carcase must be detained on the premises pending APHA advice.

It is currently advisable that animals known to have been exposed to Natural Instinct Wild Venison food, or who have been exposed to a confirmed case but are displaying no clinical

signs, should not be tested by IGRA for at least four weeks after the last known exposure. The time required for cats to mount a detectable T-cell response to *M. bovis* is unknown as no controlled challenge studies have been performed in this species; therefore the estimate of four weeks is extrapolated from bovine data where such time-course challenge studies have been conducted (Rhodes *et al.* 2000). If first line tests are positive then diagnostic imaging should be undertaken to assess for structural (*i.e.* active) disease; this should comprise full body computed tomography (CT) scan or thoracic radiographs and, ideally, abdominal ultrasonography or alternatively, abdominal radiographs. There is currently no evidence on which to base the appropriate course of action for animals that are IGRA positive but lack evidence of structural disease. It is likely appropriate in these cases to closely monitor body weight, condition score and resting respiratory rate, and to investigate further if these change. Alternatively, some owners may elect to give prophylactic therapy; isoniazid (which is occasionally used in humans for this purpose) can be given for six months, but experience of its use in cats is limited, it has a narrow therapeutic index (in humans and mice) and therefore toxicity may occur (El-Sayed *et al*. 2010; Fernandes, G. *et al.* 2017). Instead, owners may choose to treat these animals using the triple combination of anti-tuberculous therapy (above) for three months, given that the use of this protocol is well established and the potential side effects are well documented (O'Halloran and Gunn-Moore, 2017). In this outbreak, the commonly reported side effects (defined as an incidence of between 1 in 10 and 1 in 100 patients) to treatment included pruritic skin erythema caused by rifampicin and/or neutropenia induced by chronic fluoroquinolone administration.

4.8.5 Public Health Considerations

The incidence of zoonotic transmission of *M. bovis* from cats is very rare with only six cases reported in the literature (Lewis-Jonsson, 1946; Isaac, *et al.* 1983; Une and Mori 2007; Roberts *et al.* 2014). However, there may be particular risk to owners who have compromised immune function such as those receiving chemotherapy, radiotherapy or tumour necrosis factor alpha (TNF-α) inhibitors (Hofland, *et al.* 2013; Simonsen, *et al.* 2017). Therefore, owners of cats with

active disease should be advised to seek medical advice if they are at elevated risk and/or if cats have lesions that are discharging (purulent) material or if they have a productive cough.

Owner screening tests (QuantiFERON Gold™ IGRA and/or tuberculin skin tests) conducted to date have identified likely latent tuberculosis infections (LTBI) in four owners and one vet. All of these people had been exposed to severe feline cases with purulent lesions, however, it is unclear at this time if this represents zoonotic transmission from these cats or if these individuals were infected at an earlier time. Public health agencies in the UK have previously assessed the risk to immunocompetent owners from *M. bovis* infected pets and have concluded that there is 'very low' risk of zoonotic transmission (Public Health England, 2014).

4.9 Epidemiological Investigation

In general terms, the only common factors linking these cases were the raw cat food consumed (Natural Instinct, Wild Venison) and being mostly indoor cats.

Two breeding colonies were affected; one in the north of England had three clinical cases, two IGRA positive cats and two IGRA negative cats in the colony. Tracing rehomed kittens identified ten with clinically signs and two that were IGRA positive but clinically well. The second breeder, located in the south of Scotland, had eight breeding cats of which one was clinically sick and four were well but IGRA positive. Ten kittens from two litters rehomed from the second breeder were traced; half of these were tested by IGRA and all five were negative. There were no other common associations between the remaining cats.

The alimentary tract and associated lymphoid structures were found to contain lesions in 31 of the 45 cases (68.9%) which is consistent with challenge via the gastrointestinal route, supporting the hypothesis of food as the common source of infection. Five cats presented with large lung abscesses, which could be associated with spread via the retropharyngeal lymph nodes, inhalation of *M. bovis* from contaminated food during eating or may represent dissemination of disease.

All cases were presented to their primary veterinary surgeons between July 2018 and July 2019. A working estimate for the incubation period is two and five months for alimentary infections of tuberculosis in cats. This estimate is based on experimental data from the 1900's presented by Francis (1958) in which very large doses (1-10mg) of *M. bovis* when given by mouth and produced lesions 40 to 121 days later. However, extrapolating for more advanced pathology and the potentially lower doses encountered in any contaminated raw pet food could extend this significantly. Beyond this, the kinetics of feline *M. bovis* infection following oral challenge are unknown; however, it is likely that in at least some cats, a long sub-clinical phase is seen, as in cattle and humans (where it is termed latency), and this may extend the upper range of incubation to many months or even years.

Identifying a likely infection window and determining whether the cats were all infected by one particular batch of contaminated food are difficult questions to answer. The earliest signs were reported in the first weeks of July 2018; if a minimum incubation period is approximately two months, then a contaminated batch of raw pet food manufactured in early May 2018 would be implicated. With the apparent last cases being identified in July 2019, this theoretically includes all batches of food made as late as January 2019 when the production of this product was ceased by Natural Instinct. The age of the cats affected limits the potential infection window to some degree; excluding kittens weaned directly onto Natural Instinct Wild Venison, the youngest case was one year old when signs were first seen in August 2018, which extends the potential time of potential challenge back into 2017.

With the possibility of long sub-clinical chronic infections adding a considerable degree of open-endedness to the upper incubation period range, contaminated batches could theoretically have been produced during the whole of 2018, possibly 2017, or even earlier.

The temporal clustering of the majority of the early cases from July to September 2018 suggests a most likely possible common infection window of February to July 2018 (calculated from a most likely incubation period range of two to five months). Subsequent cases therefore either include a period of sub-clinical infection or represent the later consumption of stored food. The breeder in the north of England was clear about the infection window for their cats;

it had to be limited to June 2018 as this was the only time the cats had been fed Natural Instinct, Wild Venison.

An important factor that potentially explains the extended period of this cluster is long storage of the pet food in home freezers. Since Natural Instinct, Wild Venison was marketed with 'Limited Availability' owners reported that they tended to bulk purchase when it was available. The freezer shelf life was several months (calculated as nine months from data on packaging) and so a single contaminated batch could feasibly be responsible for this whole cluster.

Overall, the most likely infection window was probably March to June 2018 and all cases associated with this raw pet food could plausibly fit within this window, with stored food and sub-clinical incubation giving rise to later cases. The estimated two to five month incubation period is supported by the currently observed decline in the presentation of new cases following the recall of the food in December 2018.

Transmission to the cats from the local environment was considered to be very unlikely because a) the cats were kept indoors, with a few cats having limited usually supervised outdoor access, and b) the majority of cases were located in the LRA *for endemic M. bovis*. Infected vermin entering the houses cannot be ruled out for cases in only two households located in higher incidence bovine tuberculosis areas. However, they were not in regions with endemic *M. bovis* genotype 10:a infections.

A contaminated raw commercial cat food (Natural Instinct, Wild Venison) is the most likely route of infection for these cats. However, alternative pathways cannot be completely ruled out. Most owners of pedigree cats were located in the LRA and obtained kittens from within the LRA, but other cats in the same households had prior ownership which could not be fully investigated, so they could have had previous outdoor access in the HRA. Some of the non-pedigree cats had vague histories, including an in-contact cat that was obtained from a website with no history at all. Incomplete information creates some uncertainty for several of the cases and failure to confirm infection by the gold standard test for the majority of cases adds a degree of uncertainty as to the true number of *M. bovis* cases seen.

Within this group of 45 clinical cases, *M. bovis* genotype 10:a has been cultured from five cases from different areas of England and Scotland, with an additional Scottish sample where culture is still pending. Whole genome sequencing of the five *M. bovis* 10:a isolates by APHA Weybridge found them to be identical to the nucleotide level, *i.e*. they were identical isolates of *M. bovis*, which provides very strong evidence in support of the dietary infection hypothesis. Furthermore, the nearest cattle isolates with only one single nucleotide polymorphism (SNP) difference form a tight cluster of six farms in Oxfordshire. An *M. bovis* isolate from at least one of two wild fallow deer shot in this area had an identical genome sequence to these cattle isolates suggesting that wild deer from this area may be the origin of contaminated venison. Precise information detailing the source of venison meat and offal is needed to definitively tie the pet food and deer from this area together which is the subject of an ongoing investigation by the Food Standards Agency (FSA) and the local authority. However, an example record list for a known venison supplier to the pet food manufacturer includes one estate in west Buckinghamshire that is very close to the farm cluster and provides a tentative but plausible risk pathway for these cat cases consistent with the whole genome sequence analyses.

Further analysis of pet food obtained from owners could provide definitive evidence of the transmission route. However, batches matching the earlier period of the most likely infection window are unsurprisingly not available and no *M. bovis* has been isolated from the few samples tested by APHA, the FSA or the University of Edinburgh to date.

During this investigation, *M. bovis* was cultured from additional cats that were fed Natural Instinct Wild Venison and were euthanased, often with severe mesenteric lymph node pathology. However, the cultured isolates were found to be 11:e and 25:e. Therefore, there were thought to most likely represent alternative sources of infection. Conversely, *M. bovis* genotype 10:a was isolated from a cat during the timeframe of this outbreak which triggered a further investigation but this cat was located within the homerange of this genotype, had a superficial skin lesion consistent with inoculation via a bite wound and had no history of being fed a raw food diet so was excluded from the group of outbreak cases.

4.10 Discussion

This cluster of cases represents the only documented outbreak of feline tuberculosis putatively caused by feeding pet cats a contaminated commercial raw food diet. Previous examination of feline mycobacterial cases in the UK showed the highest frequency of infections in non-pedigree, male cats with frequently reported hunting behaviour that then develop skin lesions, probably following inoculation of mycobacteria directly into the site of the lesion (Gunn-Moore *et al.* 2011a, Gunn-Moore 2014). By contrast, the majority of cases affected in this outbreak were pedigree or pedigree-cross breeds (86.7%) and there was no statistically significant predilection for males to be affected.

The reason for the high proportion of pedigree cats affected is likely that owners of these cats are more likely to feed raw food diets. Although this has not been studied systematically, anecdotal evidence suggests that a growing number of pedigree cat breeders in the UK (and elsewhere) are advocating feeding so-called biologically appropriate raw food (BARF) diets to owners acquiring kittens (Handl, 2014; Waters, 2017). The evidence supporting the use of these diets remains poor despite their increasing popularity in recent years (Schlesinger and Joffe, 2011; EFSA, 2015). There have been speculative suggestions that BARF feeding may be beneficial in preventing the onset of dental disease, may alleviate food responsive inflammatory bowel disease and atopy, and a recent study demonstrated that the microbiome of dogs fed BARF diets is more diverse than when fed processed diets - though no health benefits were identified as a result of this diversity (Handl, 2014; Sandri *et al.* 2017). Kittens raised on a rabbit-based raw diet had better stool quality, assessed with a visual grading system, than their cooked diet fed peers, but both groups grew similarly (Glasgow *et al.* 2002). However, some of the kittens fed the rabbit-based diet developed dilated cardiomyopathy secondary to taurine deficiency after several months of feeding (Glasgow *et al.* 2002). A critical review by Schlesinger and Joffe (2011) concluded that the evidence advanced for the claimed health benefits of raw feeding amounted to opinions and claims that were, at best, supported by data that was of low relevance.

A major and growing concern relating to feeding pets these diets are the risks posed by microbiological contamination that are not addressed by cooking. The bacteriological quality of raw commercial pet foods, assessed by total bacterial, coliform or *Escherichia (E.) coli* counts, has been noted to fail threshold levels for raw human meat products in a high proportion of sampled foods in both Europe and North America (Freeman and Michel, 2001; Towell, 2008; Nemser *et al.* 2014; Kölle and Schmidt, 2015; Nilsson, 2015). The most recent systematic review, conducted by Davies *et al.* (2019), found evidence that the bacteria in these diets often encode resistance to critically important antibiotics such as extended-spectrum cephalosporins, and raw-fed pets create an elevated risk of shedding these resistant bacteria (Nilsson, 2015; Baede *et al.* 2017; Davies *et al.* 2019). Despite this evidence, the vast majority of owners interviewed (94%) in a separate survey believed raw diets to be “absolutely” safe for their dogs (Davies *et al.* 2019). During the current investigation, health screening of exposed individuals identified four likely LTBI owners and one veterinary surgeon. Although it has not been demonstrated that these likely infections originated from handling the implicated food or their affected cats, in light of these findings, the precautionary advice against raw feeding issued by various professional bodies, such as the World Small Animal Veterinary Association, appears to be justified. Both veterinary and public health practitioners need to continue to exercise their responsibility to communicate these risks clearly to consumers of raw pet food.

More than half of clinical cases in this outbreak presented with palpable abdominal masses (granulomas) and gastrointestinal lymphadenopathy which supports the hypothesis that this outbreak was due to a contaminated food product. In a study reporting the clinical presentation of feline mycobacterial infections likely to have been acquired from hunting, the only alimentary associated sign reported was weight loss, identified in 15.8% of cases none of which had directly palpable masses (Gunn-Moore *et al* 2011a). In the current cluster, twice the proportion of affected cats (33.3%) showed declining body weight/condition. Additionally, cats in this cluster were frequently anaemic and pyrexic which suggests that there may be a more severe phenotype of disease associated with this cluster than would be expected from sylvatic infections (which are rarely pyrexic; data unpublished). This is also supported by the very high

mortality rate reported for the early cases (O'Halloran *et al.* 2019). The reasons for this are not immediately clear; it may be that these cats were exposed to the largest amount of contamination, or it may be that these cats, being largely pedigree breeds, responded differently to mycobacteria; for example, susceptibility to *M. avium-intracellulare* complex infection and disease has previously been reported in Abyssinian cats (Baral *et al.* 2006). Alternatively, it may be that genotype 10:a of *M. bovis* is particularly virulent in the context of companion animal infections; for example, when the same genotype infected a group of Foxhounds it caused fatal fulminant infections in a number of animals (O'Halloran *et al.* 2018b). Further work investigating the host-pathogen interaction of *M. bovis* strains and companion animals is needed to examine this further as it may be significant in predicting patient prognosis and qualifying zoonotic risk to owners.

Many of these cats presented with respiratory signs that was unexpected. The presence of individual demarcated granulomas (as shown in Figures 4.12a and 4.12b) appears to be most consistent with primary inhalation of bacilli into the lungs, presumptively as the cats were eating contaminated food. In a number of cases, the pattern of lung pathology was more suggestive of disease spreading from a separate focus (such as the gastrointestinal tract). The interstitial lung pattern seen when imaging these cases indicates likely haematogenous spread of infection and influx of inflammatory cells into the lung parenchyma. Importantly, many of the IGRA-positive cats with no detectable respiratory signs on owner history or clinical examination were found to have thoracic pathology on imaging, highlighting the need for veterinary surgeons to investigate cases thoroughly if there are concerns over potential exposure.

The epidemiological investigation into this outbreak has demonstrated that the most likely source of infection of these cats was the consumption of a raw food diet (Natural Instinct, Wild Venison) and that one contaminated batch could have potentially been responsible for all of the cases diagnosed. In order to increase the certainty of these investigations it would be beneficial to have a greater number of samples submitted for the gold standard test of mycobacterial culture as genotyping and sequencing isolates adds significant clarity. However,

this was hindered here by the lack of state funding for most companion animal samples so that the cost to owners for official confirmation of infection through APHA are now estimated to be in excess of £500 (including *post mortem* examination). Many owners saw this as prohibitive and there was significantly greater uptake of this option when the decision was taken by APHA to relax the eligibility criteria for funded submissions after the initial cases presented. As an alternative, owners chose to use non-validated but less costly tests; further work to validate these tests would be beneficial for the investigation of any future outbreaks.

Definitive proof that the implicated raw food was the source of infection in these cats would require the isolation of viable *M. bovis* organism from a sample of the food from the at-risk period. However, a small number of samples have been tested to date and have been found to be negative. This is likely to be because the food samples submitted were from the time that the cats were diagnosed rather than the point at which they were infected. Additionally, the bulk purchasing of food by owners further creates problems for test sensitivity when large batches of food need examining.

In conclusion, this is the first report of an outbreak of *M. bovis* tuberculosis in pet cats where there is compelling evidence that a contaminated raw food product was the origin of infections. All culture positive tissues had a genetically identical genotype of *M. bovis* isolated, which has not been detected in the areas local to the cats. Based on our estimates of incubation times and from the epidemiological investigation, it is possible that a single batch of contaminated food produced in late 2017 or early 2018 resulted in the whole cluster of cases. Four cat owners and one vet were diagnosed with suspected latent TB, potentially due to exposure to infected cats with purulent lesions and/or their contaminated feed, though this remains unproven. Though the risk posed by *M. bovis* infected cats to humans is low, this outbreak demonstrates that clinicians and pet owners must continue to be made aware of the microbiological risks of raw food diets to themselves and their pets.

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Chapter 5: An outbreak of tuberculosis due to *Mycobacterium bovis* infection in a pack of English Foxhounds (2016-2017).

Preface

The following Chapter presents the findings of a study, conducted as part of this studenship programme that has been published in the journal *Transboundary and Emerging Diseases.*

The manuscript derived from this Chapter is available as an eponymous Open Access article: O'Halloran, C., Hope, J.C., Dobromylskyj, M., Burr, P., McDonald, K., Rhodes, S., Roberts, T., Dampney, R., De la Rua-Domenech, R., Robinson, N., Gunn-Moore, D.A. An outbreak of tuberculosis due to Mycobacterium bovis infection in a pack of English Foxhounds (2016–2017) (2018) *Transboundary and Emerging Diseases,* **65 (6),** 1872-1884.

Author contributions:

C. O'Halloran, the candidate, conducted the investigation, diagnostic testing, performed the data analysis and wrote the manuscript.

Dr Dobromylskyj diagnosed the Index case. Dr Burr and Mr McDonald provided laboratory facilities and assisted the candidate with performing the IGRA tests described herein. Dr Rhodes provided expert advice on developing mycobacterial testing protocols for new species. Due to difficulties in obtaining DPP tests commercially, Dr Rhodes ran those assays and provided the results to the candidate for analysis and interpretation. Professor Hope provided assistance with Containment Level 3 work. The Animal and Plant Health Agency team (Roberts, Dampney, De la Rue-Domenach and Robinson) provided advice and support to the testing protocol and assistance with the epidemiological investigation. Professor Gunn-Moore supervised the project.

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Abstract

Mycobacterium bovis can cause tuberculosis in social mammals including lions, cattle and man, but canine infections are considered rare.

In 2016/17 we investigated a *M. bovis* TB outbreak in a pack of approximately 180 Foxhounds within the bovine tuberculosis Edge Area of England. We employed a combination of

immunological tests including an interferon gamma release assay (IGRA) and a serological assay (DPP VetTB, Chembio). Test-positive hounds were euthanased and subjected to *post-mortem* examination (PME).

Overall 164 hounds were tested; 97 (59%) responded positively to at least one test. Eighty-five (52%) dogs responded to *M. bovis* antigens by IGRA whilst only 21 (12.9%) had detectable serological responses. At PME three hounds (3.1%) had visible lesions (VL) due to *M. bovis* infection, later confirmed by culture. Samples from 24 non-VL hounds were cultured and *M. bovis* infection was confirmed in a further three hounds (11%).

This study is the first investigation and report of an outbreak of *M. bovis* TB in a canine species. We establish that, in principle, diagnostic tests used for identifying infected individuals of other species can effectively be used in the dog. Further work is urgently needed to establish the sensitivity and specificity of the testing approach used in this study for future clinical application.

5.1 Introduction

Mycobacterium (M.) bovis is one of the nine member species of the *Mycobacterium tuberculosis* complex (MTBC) which are capable of causing tuberculosis, across a broad taxonomy of social mammal including but not limited to humans, lions, elephant and meerkats (Parsons, 2013; Angkawanish, 2013; Miller, 2012; Drewe, 2010; Brosch, 2002). Within this group of pathogens, *M. bovis* stands out as the least host restricted and thus has significant zoonotic potential (Miller, 2013; Michel, 2013; Broughan, 2013; Palmer, 2007). Disease in humans due to infection with *M. bovis,* termed ‘zoonotic tuberculosis’ by the World Health Organisation (WHO), is a major global public health priority which resulted in nearly 150,000

cases and at least 12,500 deaths in 2010 worldwide (Olea-Popelka, 2017; Dürr, 2013). Additionally, the lack of diagnostic discrimination between *M. tuberculosis* and *M. bovis* in some human cases by reference laboratories means that the true mortality and morbidity caused by zoonotic tuberculosis is possibly underestimated (Olea-Popelka, 2017; Dürr, 2013).

Discriminating between tuberculosis caused by different species of the MTBC is challenging as all member species share identical sequences across the 16s rRNA gene and 99.5% sequence homology across the remainder of the genome (Brodin, 2002). The most discriminating features between the species at the nucleotide level are genomic deletions, termed regions of difference (RD) (Teo, 2013). These have been shown to encode a variety of different virulence factors e.g. RD-1 is present on all MTBC mycobacteria other than *M. bovis* bacille-Calmette-Guérin (*M. bovis*-BCG) and *M. microti* and encodes for the immunodominant proteins and key virulence factors; a 6kDa early-secreted antigenic target (ESAT-6) and 10kDa culture filtrate protein (CFP-10) (Junqueira-Kipnis, 2006; Guinn, 2004; Gao, 2004).

The human burden of disease is highest across Africa, South-East Asia and the Western Pacific. In South Africa, dogs have been shown to become infected with *M. tuberculosis,* as diagnosed using human interferon gamma release assays (IGRA), when exposed to high risk humans i.e. those with active tuberculous disease (Parsons, 2012). Similarly, African wild dogs (*Lycaon pictus*) have been found to be infected with *M. bovis,* presumptively from hunting exposure (Ayele, 2004). In the UK, however, the significance of *M. bovis* in companion animals is largely limited to domestic cats, were frequent diagnoses are made (Pesciaroli, 2014; Gunn-Moore, 2014; Broughan, 2013). Canine incidence of tuberculosis in the UK is currently considered to be rare, and almost all reported cases are limited to individual sporadic infection or small numbers of epidemiologically unrelated cases (Szaluś-Jordanow, 2016; Park, 2016; Pesciaroli, 2014; Parsons, 2012; Posthaus, 2011; Van Der Bürgt, 2009; Shrikrishna, 2009; Ellis, 2006; Gay, 2000; Liu, 1980; Snider, 1971).

Between the end of 2016 and July 2017, a large outbreak of *M. bovis* infection occurred in a kennel of working Foxhounds within the Edge Area of England; which is a buffer zone of intermediate bovine tuberculosis incidence separating the high and low risk bovine tuberculosis areas and subject to additional surveillance and controls for this disease. This report describes the clinical features of the outbreak, the testing approach taken and the epidemiological aspects of the spread of disease as they are currently understood. Work to evaluate the testing approach further is currently ongoing and will be published separately (Chapters 7 and 8). We go on to explain the new statutory controls which have been implemented in England as a result of this incident to minimise the low risk of spreading tuberculosis through the feeding of fallen stock to hounds in registered kennels.

5.2 Outbreak Investigation

5.2.1 The Index Case

The hunt kennel where this outbreak occurred is situated in the south of England, UK, and housed up to 180 working Foxhounds ranging in age from juvenile puppies (less than ten weeks old, n=32), young adults (up to and including a year old, n=21) and adults up to eight years old (median age of four years).

The kennel is situated within the designated Edge Area of bovine TB incidence. The hounds work across six counties, four of which are also within the Edge Area, and two in the Low Risk Area, up to three times weekly during the peak of their hunting season (August to April).

In a similar fashion to many hunt kennels, the hounds were predominately fed raw meat, permitted offal and bone from fallen stock (so called “flesh feeding”), as permitted under Animal By-Products legislation (Article 18, Commission Regulation (EC) No. 1069/2009).

The index case (Case 0) occurred in December 2016. The pack had experienced an outbreak of upper respiratory tract disease in the previous weeks, resulting in a number of individual hounds suffering from reduced body condition, lethargy and poor appetite. Two of these hounds developed marked polyuria and polydipsia and died. Clinical suspicion at this time was of leptospirosis infection as routine vaccination is not used and clinical signs were compatible (Rissi, 2014). The hounds were not subjected to any confirmatory diagnostic testing at this time and carcasses were disposed of. When a third hound, a four-year-old entire male Foxhound with no previous history of ill health, developed anorexia, plus marked polyuria and polydipsia, blood samples were taken for routine haematology, serum biochemistry and leptospirosis IgG titre testing.

These tests revealed; an inflammatory leukocytosis ($21.8x10^9$/L [reference interval, RI, 6-$15x10^9$/L]) consisting of a mature neutrophilia ($17x10^9$/L [RI 3.6-$12x10^9$/L]), lymphopenia (0.4 $x10^9$/L [RI 0.7-$4.8x10^9$/L]), monocytosis ($3x10^9$/L [RI 0-1.5 $x10^9$/L]) and eosinopenia ($0x10^9$/L [RI 2-10 $x10^9$/L]). Serum biochemical analysis showed a marked azotaemia (urea 18.7mmol/L [RI, 1.7-7.4mmol/L], creatinine 457µmol/L [RI 40-132µmol/L]), hypoalbuminaemia (18g/L [RI 26-35g/L]) and hypocalcaemia (total calcium 2.04mmol/L [RI 2.30-3.00mmol/L]). Serological testing returned only a very low positive combined leptospirosis IgG titre (1+), indicating that active leptospirosis was unlikely to be the cause of the azotaemia and other clinical signs.

The dog was euthanased and subjected to *post mortem* examination (PME). Both kidneys were found to be grossly diffusely grey in colour and firmly textured. Across both the visceral and cut surfaces were multifocal to coalescing nodular, approximately round, areas containing

hard white and soft black material (Figure 5.1). Representative samples were collected and fixed in formalin for histological evaluation by one of the authors (MD, FRCPath and experienced veterinary pathologist). Additional samples were frozen and submitted for mycobacterial culture and PCR.

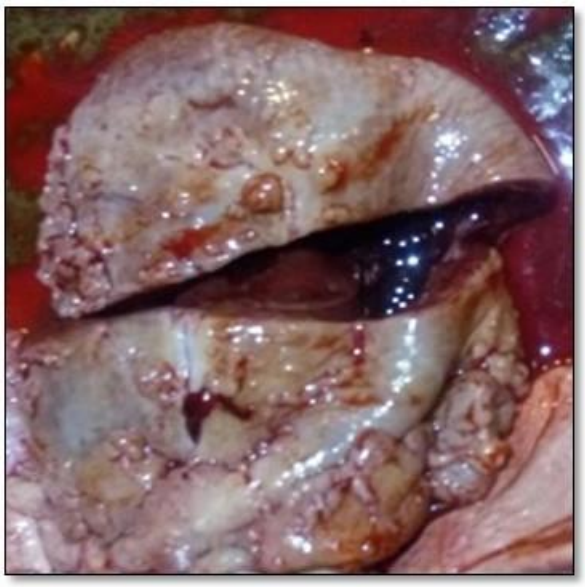

Figure 5.1: The left kidney of Case 0, removed at PME, the visceral and cut surfaces show diffuse discolouration and there are observable granulomas on the visceral surface.

Examination of haematoxylin and eosin stained sections showed extensive, multifocal to coalescing areas of severe, chronic-active, necrotising, granulomatous to pyogranulomatous nephritis (Figure 5.2). Silver staining for the identification of spirochetes was negative, as was periodic acid-Schiff staining for fungi (not shown), while a Ziehl-Neelsen stain revealed moderate numbers of positive staining (acid-fast) bacilli morphologically typical of mycobacteria within the cytoplasm of epithelioid macrophages, indicative of mycobacterial infection (Figure 5.3).

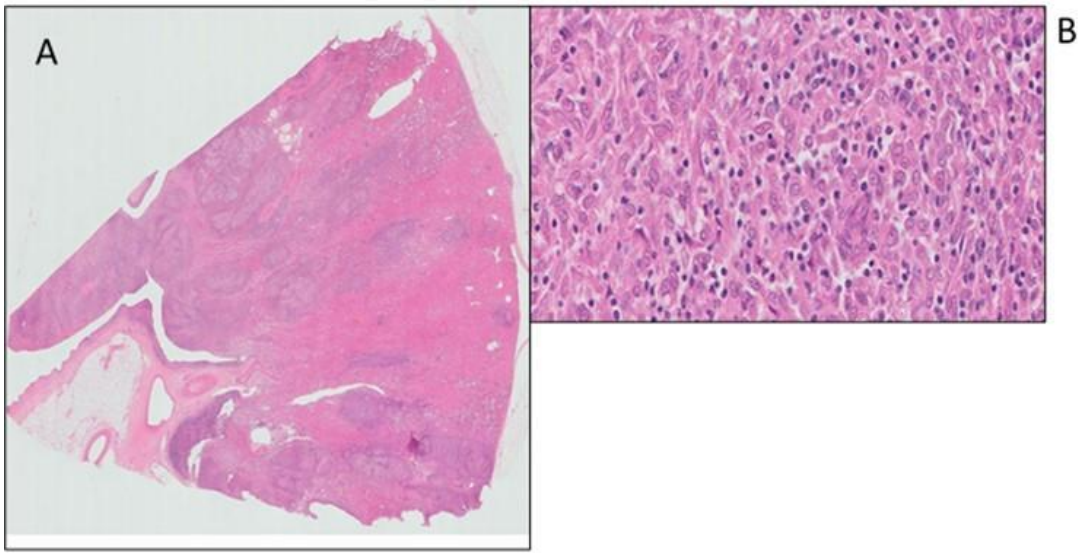

Figure 5.2: A section of kidney from case 0 (shown grossly in Figure 7.1), stained with haematoxylin and eosin. Image A shows the section at low power (x10 magnification) where multifocal areas of pathology (granulomas) are visible. Image B is a high power view of the same section (x40 magnification) and shows numerous epithelioid macrophages with neutrophils. The kidney is affected by severe, chronic active necrotising granulomatous to pyogranulomatous nephritis.

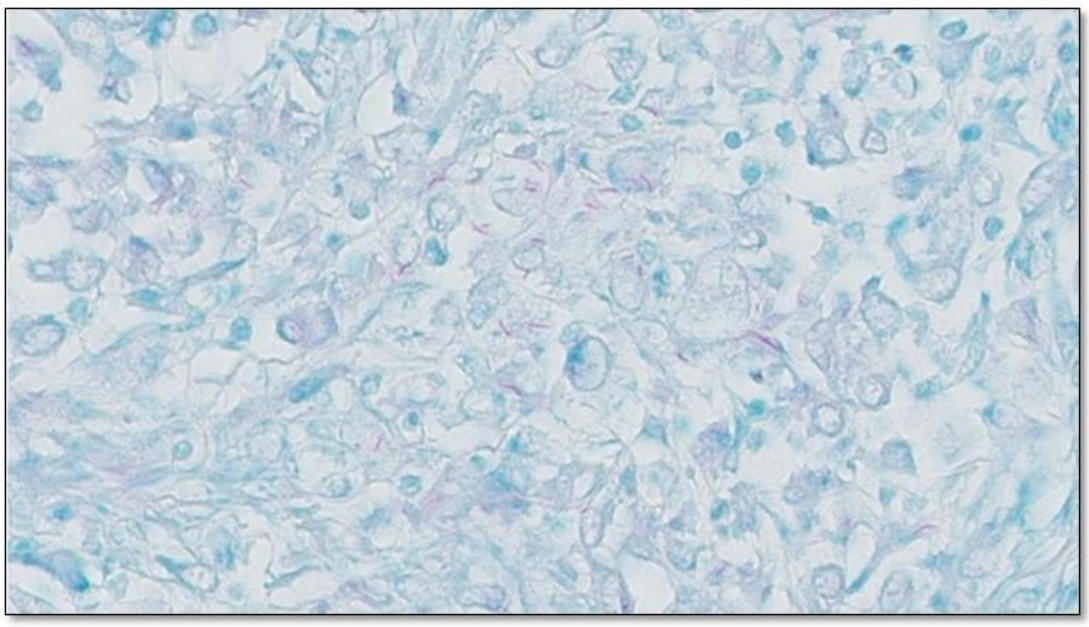

Figure 5.3: A section of kidney from Case 0 stained with Ziehl-Neelsen stain shown under oil emersion x100 magnification which reveals moderate numbers of intracellular and extracellular acid-fast organisms morphologically typical of *Mycobacteria spp.*

The Animal and Plant Health Agency (APHA) was notified of the findings and remaining fresh tissue was submitted for both routine mycobacterial culture (APHA, Weybridge) and for mycobacterial PCR (Leeds University Mycobacterial Reference Laboratory [LUMRL]), both of which confirmed infection with *M. bovis* subsp. *bovis* which was genotyped to 10:a.

5.2.2 Testing Regime

Once *M. bovis* infection had been confirmed a prospective test and cull policy was implemented at the kennels in order to contain the spread of infection within the pack and remove any potentially infected animals. This was combined with immediate voluntary movement restrictions implemented by the kennel and increased biosecurity measures e.g. increased kennel disinfection protocols, cessation of feeding fallen stock and immediate repairs to limit wildlife access to the hounds.

a) Interferon-gamma (IFN-γ) release assays (IGRA)

IGRA tests were originally developed on the principle of quantitatively evaluating IFN-γ production by peripherally circulating antigen-specific effector memory T-cells upon *in vitro* stimulation, in order to aid the diagnosis of *M. bovis* tubserculosis in cattle, where it has a reported sensitivity of 81.8-100% and specificity of 88-99% (Vordermeier et al., 2006; Schiller et al., 2009; Bezos, 2014; Wood, 2001). The IGRA has subsequently been adapted to identify active and latent tuberculosis in human patients with at least equivalent sensitivity and increased specificity than the tuberculin skin test, as well as being practically easier to perform (Thillai, 2014; Eisenhut, 2014; Zhou, 2011; Kim, 2011). An IGRA test has been validated for use in pet cats with up to 100% sensitivity for the detection of MTBC infections (Rhodes, 2011; Rhodes, 2008a; Rhodes 2008b). Whereas intra-dermal testing has been shown to be of unreliable clinical utility in the dog, an IGRA has been used successfully for the detection of *M. tuberculosis* infections in dogs in a high risk setting (Parsons, 2012). The *in vitro* nature of

the test allowed us to test individual animal responses to multiple antigen combinations with technical replicates.

To interrogate the immune response of the hounds to mycobacteria, the antigens purified protein derived (PPD) from *M. avium* (PPDA), PPD from *M. bovis* (PPDB) and a cocktail of the RD-1 specific immunodominant proteins ESAT6 and CFP-10 were selected. The PPDA is frequently used in both IGRA and tuberculin skin testing to assess for exposure/sensitisation to, or infection with, environmental mycobacteria species (Rhodes, 2008a; Pai, 2007; Wood, 2001). Infection with an MTBC mycobacteria is confirmed if the IFN-γ response of any animal is greater to PPDB than to PPDA (Rhodes, 2008a; Wood, 2001). The presence of a concurrent response to the immunodominant antigens ESAT-6/CFP-10 indicates infection with an RD-1 positive MTBC mycobacteria (i.e. excludes infection with *M. microti* or previous vaccination with *M. bovis*-BCG) (Teo, 2013; Guinn, 2004).

The IGRA assays were conducted at Biobest Laboratories, Edinburgh in line with the assay protocol validated and previously published for use in the cat (Rhodes, 2011; Rhodes, 2008a). A 5ml heparinised whole blood sample was taken from each hound and transported to the laboratory at ambient temperature within 18 hours. Upon receipt, peripheral blood mononuclear cells (PBMC) were removed; blood was diluted 1:1 with Hanks Balanced Salt Solution (HBSS, Gibco, UK) and layered over Histopaque 1077 (Sigma, UK) before centrifugation at 800 x *g* for 40 minutes at room temperature. PBMC were removed from the resulting interface, washed with HBSS and re-suspended in complete culture media (RPMI 1640 containing 100µg/ml L-glutamine, 10% foetal bovine serum, 100µg/ml penicillin, 100U/ml streptomycin, $5x10^{-5}$M 2-mercaptoethanol and non-essential amino acids) to a density of $2x10^{6}$/ml. 100µl of PBMC suspension were stimulated in duplicate with PPDA or PPDB (Lelystad, Prionics, Netherlands); both at a final concentration of 10µg/ml, as well as a peptide cocktail of ESAT-6/CFP-10 at a final concentration of 5ug/ml (Lionex, Germany), a mitogen positive control of phorbol myristate acetate plus calcium ionophore (PMA/Ca, Sigma, UK, 50ng/ml and 1µg/ml respectively), and finally a complete culture medium only (i.e. negative) control.

Cells were incubated for 4 days at 37ºC/5% CO_2, after which the supernatants were removed, and duplicates pooled, for quantification of IFN-γ by ELISA. Supernatants were either directly assayed or stored at -80ºC until required. The IFN-γ ELISA was conducted using a commercially available canine specific ELISA kit (DY781B, R&D Systems, Europe Ltd., UK) according to the manufacturer's instructions. Supernatant from each cell culture condition was assayed in duplicate. Optical density (OD) values were measured at a wavelength of 450nm and the replicate values for each condition were averaged and standard deviations calculated. Where replicates differed by more than 30% from each other, the test was considered invalid.

Prior to the instigation of testing, a prospective case definition was set to determine which hounds would be considered positive; it was decided that a statistically significant response to any of the three test antigens (PPDB, PPDA or ESAT6/CFP10) above the negative (medium control) condition was plausibly indicative of a biologically significant antigen specific T-cell response. Furthermore, such a response would be consistent with not just exposure to mycobacteria but rather would be suggestive of significant challenge and probable infection. To achieve maximum diagnostic sensitivity, and due to the fact that very little is known about the infection dynamics, clinical progression or probability of mycobacterial shedding by dogs once they are infected with mycobacteria, it was assumed that any hound with a significant IFN-γ response to at least one test antigen was infected and potentially infectious. Any such hound would therefore pose a risk to human and animal health as well as to the environment and so should be removed from the pack and euthanased.

As no cut off values or reference intervals exist for the dog, we defined the threshold for responsiveness to antigen stimulation as the mean OD value of the negative control replicates, plus two standard deviations ($\bar{x} + 2SD$). Antigen responses were defined as the mean OD value of the replicates, minus two standard deviations ($\bar{x} - 2SD$). If a calculated antigen response value was greater than that calculated for the negative control, then the response was considered positive. For a test to be considered interpretable, the PMA/Ca (positive control) response had to be positive by the same criteria when compared to the negative control.

In total 164 hounds were tested by IGRA, only 11 tests failed and required repetition; the most frequent reason for this (n=6) was that there was insufficient response to the PMA/Ca positive control, followed by a clotted blood sample which precluded the isolation of PBMC (n=4) and only one test required repetition due to significant disagreement between replicate values. All IGRA test result data are provided in Appendix 1.1.

Of the 164 hounds, 85 (52.0%) were found to be positive by the above case definition. Within those found to be positive, 77 (90.1%) displayed a significant response bias to PPDB which was greater than the PPDA response (which was also significantly elevated above the negative condition in 37, 48.1%, of these hounds), a pattern considered indicative of MTBC infection.

Within the group of hounds displaying a typical MTBC infection response, 48 (62.3%) also responded to the peptide cocktail ESAT-6/CFP-10. Five of the test positive individuals (3.1%) responded to ESAT-6/CFP-10 and no other test antigens. These individuals were considered positive by the case definition and so were removed from the pack and euthanased. Similarly, only three individuals (1.8%) responded to PPDA and no other test antigens; whilst it was considered unlikely that these were infected with MTBC mycobacteria, they were removed from the pack, and euthanased, as a precaution.

b) Serological testing

Concurrently to the IGRA testing, serological assessment of each hound was conducted. Blood was taken from each hound at the same time as the whole blood was collected for IGRA testing, 5mls of whole blood was placed into plain blood tubes and left upright at room temperature to allow clot formation. On arrival at the laboratory, blood was centrifuged at 800 x *g* for 15 minutes at room temperature. Serum was removed and frozen at -20^{o}C in 500µL aliquots until needed.

The Dual Path Platform (DPP) VetTB test for Cervids (Chembio Diagnostic Systems Inc., USA) consists of two nitrocellulose strips inside a cassette that allows independent delivery of the test sample and antibody-detecting reagent to the two test antigens (MPB83 and combined

ESAT-6/CFP-10) and the test control. One cassette was used per hound. Tests were carried out at APHA Starcross. Thirty microliters of serum was dispensed into the sample well followed by two drops of sample buffer. After five minutes, a further four drops of sample buffer was added to the buffer-only well. Cassettes were incubated for a further 20 minutes. This protocol is used by APHA for Eurasian badger (*Meles meles*) with preliminary test estimates of test sensitivity of 55.3% (38-71.4%) and specificity of 97.5% (86.8-99.9%).Test results were obtained by inserting each cassette into a hand-held optical reader device (Chembio Diagnostic Systems Inc., USA), measuring reflectance in relative light units (RLU). An RLU numerical value for the control band (to show the test was valid) and the test band was provided by the reader.

Test results were obtained first qualitatively by visual inspection and to check the cassette quality control band and the presence/absence of antigen binding by serum antibody, and then quantitatively by inserting each cassette into an Optricon DPP Reader (Chembio Diagnostic Systems Inc., USA), to measure reflectance of antigen binding by antibody in relative light units (RLU).

A total of 164 hounds were tested; of these 11 (6.7%) generated positive test bands visible by eye; of these, nine had antibodies directed against antigen MPB83 and two against ESAT-6/CFP-10. No hounds were positive to both MPB83 and ESAT6/CFP10. Hounds which produced a visibly positive test were considered to be infected, removed from the pack and euthanised.

Additionally, a further 10 hounds (6.1%) showed RLU values greater than that of the majority of the remaining population and were assigned as “intermediate positive” test results (Figure 5.4). Whilst interpreting these results is challenging, these hounds were considered to be at increased risk of infection in comparison to the background population and so were also removed from the group and euthanased. All test result data for the Chembio DPP VetTB test are provided in Appendix 1.2.

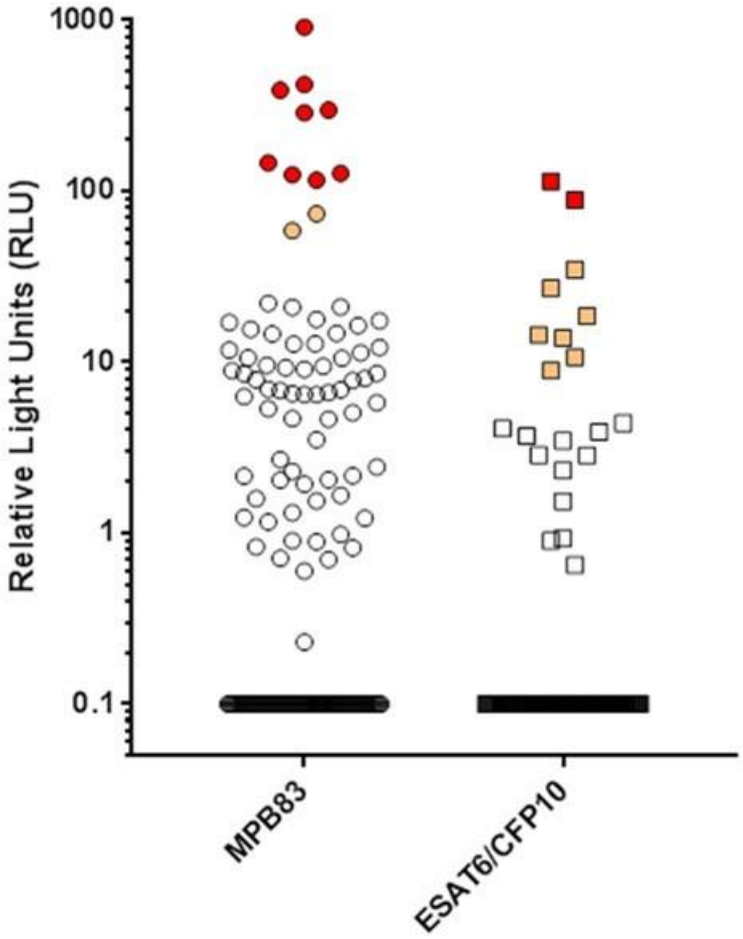

Figure 5.4: Dual Path Platform (DPP VetTB, Chembio, USA) serology test results (given as Relative Light Units [RLU]) for each of 163 hounds tested. Responses (RLU) to the two test antigens MPB83 (circles) and ESAT6/CFP10 (squares) are shown. Hounds producing a visible/qualitative test-positive result are represented by red symbols, those giving an intermediate result are represented by orange symbols and test negative animals are shown as black open symbols. Negative samples scoring zero RLU were assigned as “0.1” to allow their appearance on this log-scale graph.

5.2.3 Screening of at risk, in-contact dogs

During the course of the outbreak, a number of dogs were identified out with the pack which had considerable potential for exposure to the hounds at the affected kennels. These dogs fell into three groups; (1) those dogs kept as pets by individuals who lived at or worked closely with the kennels and who spent a considerable amount of time with the hounds, (2) individual bitches from the affected kennel, that had been sent within the previous 18 months to other kennels for the purpose of breeding and remained at those kennels, and (3) a group of bitches that were sent from the affected kennel during the 18 months prior to the outbreak, to other kennels for purposes of breeding and were then returned, possibly bringing the infection with them.

After consultation with owners, 19 at-risk pet dogs (group 1) were subjected to IGRA testing according to the same protocol as the hounds, outlined above (Section 7.2.2 sub-section a). Of these dogs only two were found to produce significant responses to PPDB and with a PPDB>PPDA bias and neither of were responsive to ESAT-6/CFP-10. Retrospective investigation revealed that one of these dogs was a hound which had been retired from the affected pack due to age-related poor performance only a few weeks prior to Case 0 becoming clinically sick. The second was a terrier dog, used as a “cutting room scrap dog” *i.e.* it was regularly close to the preparation of carcasses before they were fed to the hounds.

In total, 13 bitches were sent from the affected kennel to one of two other kennels for breeding purposes, (groups 2 and 3); both of these kennels are in the High Risk Area (HRA) of endemic bTB of England. Seven of these bitches were still away at the other kennels at the time of testing (*i.e.* group 2), where they were assessed by IGRA and DPP VetTB test assay (as outlined above) and found to be negative on both tests. Six bitches had been off site for breeding purposes in the 18 months preceding the outbreak and returned to the kennels before the first case occurred (*i.e.* group 3). Only three of these animals were still alive at the time of the outbreak; one was found to be IGRA positive (PPDB> PPDA) but was DPP VetTB test-negative and had no visible lesions at PME. The remaining two live hounds were test negative

by both assays. The reason for, and timing of, euthanasia or death of the three untested hounds is not known as it was not recorded by kennel staff.

5.2.4 *Post-mortem* examination of test positive animals and subsequent mycobacterial culture (Table 5.1)

During the course of the outbreak, from December 2016 until July 2017, a total of eight hounds became clinically unwell. All hounds displayed similar clinical signs to Case 0 including acute onset anorexia, lethargy, polyuria and polydipsia. Only one of these hounds was subjected to IGRA testing *ante mortem* and was found to be responsive to the antigen cocktail of ESAT-6/CFP-10, the remainder had to be euthanased for welfare reasons before samples could be obtained.

All IGRA and serology test positive but clinically healthy animals were euthanased and carcasses disposed of by incineration. Prior to incineration, all carcasses were subjected to a minimum of a gross PME; the exterior of the carcass was assessed, the abdominal and thoracic cavities were opened and all viscera visually inspected. Overall, 97 of the 164 hounds tested in the initial screening tests were euthanased. Of these 97, three (3.1%) were found to have grossly visible renal pathology (visible lesions, VL) as described for Case 0; lesioned tissues were removed for mycobacterial culture and histological examination. In all cases, acid-fast bacilli were noted on histology and *M. bovis* was cultured from the fresh tissue (see below).

Additionally, 24 (25% of the 97) non-visible lesioned (NVL) hounds were randomly selected to have a full PME with samples taken for mycobacterial culture and histological examination. For each of these hounds the submandibular, pre-scapular, axillary, bronchial and mesenteric lymph nodes were removed with one (left hand side for paired nodes) or one half (for unpaired nodes) formalin fixed and the other kept in sterile containers for mycobacterial culture. Given the frequency of renal lesions in previously confirmed cases, representative internal samples of both kidneys as well as urine obtained by cystocentesis were taken for mycobacterial culture.

Post mortem samples were assessed by mycobacterial culture either at APHA, Weybridge (clinically sick animals only) or the Roslin Institute, University of Edinburgh (clinically sick and clinically well animals). Samples from two of the eight clinically sick animals (25%) were tested in both laboratories to check for culture agreement and both laboratories were able to grow *M. bovis* from both samples.

All samples for mycobacterial culture were treated according to APHA's standard operating procedure by both laboratories. Briefly, inside a Containment Level 3 (CL3) laboratory up to 20 g of tissue was homogenised, decontaminated with 5% oxalic acid or 10% sodium hydroxide, and centrifuged, the pellet was re-suspended in sterile phosphate-buffered saline (PBS) and centrifuged again. Urine was centrifuged and removed from the sediment. The homogenate or urine sediment respectively was then re-suspended in PBS and sown onto solid Middlebrook 7H11 OADC (Sigma, UK) and liquid Middlebrook 7H9 ADC (Sigma, UK) culture media.

Cultures were read at six weeks of incubation and again at 14 weeks in order to allow sufficient time required for any MTBC organism (i.e. either *M. bovis* or *M. microti)* to grow in culture if present in the tissue sample.

A total of 14 hounds (8.2%) were confirmed as infected with *M. bovis* by mycobacterial culture (Table 5.1). Of these; all eight hounds which showed signs of clinical disease were found to be culture positive at six weeks. Six hounds were sub-clinically infected; three were identified as being visible lesion positive at PME, in each case *M. bovis* was isolated from at least one lymph node, at least one kidney and from urine samples. All cultures in these animals were positive by six weeks. Three hounds were NVL at PME but were culture positive, as with the VL hounds, in each case *M. bovis* was isolated from at least one lymph node, at least one kidney and from urine samples, however, all cultures were only found to be positive at 14 weeks.

Positive cultures were harvested and heat killed before genotyping; *M. bovis* was identified on the basis of colony morphology and genotyping by APHA. Genotyping was performed using

spoligotyping (Kamerbeek et al. 1997) and VNTR typing (Exact Tandem Repeat loci A to F, Frothingham and Meeker-O'Connell, 1998). Positive cultures grown at the Roslin Institute were harvested and digested with enzymatic lysis buffer containing 20mg/ml lysozyme (Sigma, UK). Mycobacterial genomic DNA was obtained using a DNeasy Blood and Tissue kit (Qiagen, Germany) in accordance with the manufacturer's instructions. Harvested DNA was used as a template for PCR identification (as developed in Chapter 2) of the mycobacterial genes; the 65kDa heat shock protein (*hsp65*) and 16s rDNA. If these were positive further testing was conducted to look for the presence of the MTBC antigen 85-A, and the RD-1 specific gene ESAT-6. A summary of all PME and culture results is shown in Table 5.1.

Table 5.1: Results of *post mortem* investigations compared to *ante mortem* test results.

Initial screening tests					
	DPP positive	DPP "at risk"	DPP negative	TOTAL	
IGRA positive	4	5	76	86*	
IGRA negative	7	5	66	78	
TOTAL	11	10	142	164	
Repeat tests					
	Culture positive	Culture negative	Culture not performed	TOTAL	
IGRA positive	0	0	9	9**	
IGRA negative	0	0	57	57	
TOTAL	0	9	57	66	
Post mortem results (Hounds euthanased following initial screening)					
	Culture positive	Culture negative	Culture not performed	Total	Additional notes
Clinical signs, no *ante mortem* testing	7	0	0	7	Two hounds had histopathological renal abnormalities; chronic-active granulomatous nephritis.
Clinical signs; IGRA positive, DPP test not done	1	0	0	1	Significant IFN-γ response was seen to ESAT-6/CFP-10 only. Lesions present at *post-mortem* examination.
IGRA positive, DPP positive	2	0	2	4	Visible lesions present at *post-mortem* examination of culture-positive hounds.
IGRA positive, DPP "at risk"	2	0	3	5	
IGRA positive, DPP negative	1	17	58	76	
IGRA negative, DPP positive	1	2	4	7	
IGRA negative, DPP "at risk"	0	2	3	5	
IGRA negative, DPP negative	0	0	66	66	
TOTAL	14	21	136	171	

*One animal not DPP tested **PME of these nine hounds were unremarkable

5.2.5 Repeat IGRA testing protocol

Once the initial screening testing had been completed, the 66 hounds that remained were kept in isolation for 60 days. At this time, because the test accuracy of the two testing methods is unknown, the IGRA test was repeated. Nine hounds were found to be IGRA positive, none were visibly lesioned at PME.

Following these results it was decided that the remaining 57 hounds were unlikely to be infected/infectious and so voluntary restrictions on the kennels were lifted. The hounds continue to be closely monitored for any change in appetite, body weight and body condition score. All of the remaining hounds remain well at the time of writing.

5.3 Screening of at risk, in-contact humans

M. bovis is a known zoonotic bacteria. Once infection was confirmed by laboratory culture from samples taken from the hounds, Health Protection England (HPE) was informed to assess the risk to human health. An in-depth risk assessment was conducted, and contacts were stratified in to risk pool. A ‘stone in the pond’ approach was adopted for screening, with those at highest risk of exposure screened initially. This investigation and its findings are the subject of an additional study, where 11 people were screened (Phipps *et al.,* 2018). One asymptomatic exposed person has tested positive for tuberculosis on initial screening by IGRA and has since been diagnosed with latent tuberculosis. Due to the nature of the contact between this individual and the infected hounds, it remains possible but unproven that the person was infected by contact with either the hounds and/or contaminated bovine material.

5.4 Epidemiological assessment

5.4.1 Risk pathway identification

The APHA conducted an epidemiological investigation into the source of the outbreak to investigate possible transmission pathways. A qualitative risk assessment approach was used to address the likelihood of each of these routes of infection. The more probable contenders in order of likelihood were:

5.4.2 Movement of infected hounds into the kennels (inhalation/ingestion/biting)

Thirteen female hounds moved to, and six returned from, other kennels for breeding purposes during the 12 months prior to clinical signs in case 0. This included locations in the HRA that were also within the home-range for genotype 10:a of *M. bovis*, where infected carcases would have been more likely to have been fed. Within the previous three years, but prior to the 18 months investigation in Section 5.2.3; three hounds, two male and one female, moved onto the premises from three other kennels in the HRA. The likelihood of these hounds becoming infected from fallen stock at these kennels or through other transmission pathways in the HRA was assessed as medium and therefore the most likely. This likelihood was somewhat mitigated by the fact that only one of the tested bitches was positive to the screening tests, and she had no visible lesions at *PME*. However, not all animals could be followed up because of missing records or mortalities, only ten of the 16 traced animals were alive and available for testing at the time of the outbreak.

5.4.3 Feeding of *M. bovis*-infected fallen stock (ingestion)

Details of the fallen stock collected from farms and fed to the hounds in the kennels over the 12-month period prior to the first hound death were examined to provide an evaluation of the likely risk posed. A total of 24 carcases were sourced from an Approved Finishing Unit (AFU) for negative-testing cattle from bovine tuberculosis restricted farms. However, no tuberculous

lesions had been confirmed at *post mortem* meat inspection in the abattoir in any cattle from the unit over the previous three years, reflecting the fact that most were sourced from low bovine tuberculosis incidence areas *i.e.* from across the Low Risk Area and/or low incidence parts of the Edge Area. Apart from the AFU, six carcases were collected from farms with a history of TB breakdowns caused by genotype 10:a of *M. bovis* – two dating back to breakdowns in 2014 and one in early 2016 due to the purchase of one infected cow. However, none of these incidents had ongoing infection within the herds in question and all had been subject to parallel IGRA and tuberculin skin testing, with negative results, which reduces the probability of residual infection.

The feeding of fallen stock from local cattle herds with undisclosed *M. bovis* 10:a infection remains a low likelihood considering the low tuberculosis incidence in the area, annual tuberculosis testing of herds, and the herd level test sensitivity with a cut-off of one reactor. Only one farm was in a four year testing parish, but was assessed as low risk after considering its purchase history. No alpaca carcases or lungs had been fed. However, it is possible that kennel staff may not have recognised tuberculous lesions in carcases if present. The likelihood of feeding infected material to the hounds at this kennel was consequently assessed as low, but with a medium level of uncertainty regarding prevalence of carcase infection and dose-response in dogs.

5.4.4 Exposure to infected livestock or wildlife during exercising (inhalation/ingestion/biting) either directly or via *M. bovis*-contaminated environment

About 85% of the geographical area to which the hounds were exposed had low cattle tuberculosis incidence and no indication of wildlife infection. The remaining 15% had some tuberculous cattle breakdowns where APHA investigation indicated possible wildlife sources. The very infrequent reporting of *M. bovis* in farm dogs in UK suggests that farm-based transmission pathways are not very effective. Kennel staff reported that direct contact between wildlife and working hounds during exercise was unlikely. However, the worst case scenario

of an encounter with an infectious badger, or infected carcase could have been missed. The likelihood of infection whilst exercising was considered very low, but with high uncertainty associated with wildlife prevalence and general lack of quantification of the transmission pathways from the environment.

5.4.5 Exposure to infected local wildlife at the kennels (inhalation/ingestion/biting)

The kennels are located in an area of the country where cattle tuberculosis incidence is low and there is no evidence of wildlife infection. Access to the kennels by larger wildlife such as badgers was very unlikely. Likelihood was considered very low with low uncertainty.

Once in the kennels, infection appears to have spread horizontally between the hounds, suggesting that in certain conditions such as intensive housing of large packs, relatively high rates of transmission can occur within dog populations. It is known that badgers can excrete up to 100,000 *M. bovis* colony forming units (CFU) per ml of urine where kidneys are infected (Corner, 2012). Dogs with similar pathology may have similarly high excretory rates. Inhalation of infected urine aerosols or ingestion of such high doses are valid and likely transmission pathways in this scenario to account for dog to dog spread. However, the fact that no gross tuberculous pathology was found in the respiratory and digestive systems of the clinical cases or test positive dogs adds a level of uncertainty as to what the exact transmission route was.

5.5 Statutory changes resulting from the assessment

This outbreak prompted a policy review of the feeding of raw flesh from fallen stock to hounds, permitted under Article 18 of Commission Regulation (EC) No. 1069/2009. A condition of this regulation is that fallen stock fed are not killed or have not died as a result of the presence or suspected presence of a disease communicable to humans or animals. An interpretation of this is that TB reactors, normally removed by APHA, and inconclusive reactors, should not be fed. The Department for Environment, Food and Rural Affairs (DEFRA) commissioned a risk assessment from APHA to consider the likelihood of *M. bovis* infection occurring in hounds at

Animal By-Products (ABP) registered kennels through the routine feeding of fallen stock sourced from farms in the HRA of England.

The overall likelihood of at least one foxhound becoming infected with *M. bovis* within the next five years, if current practices remained unchanged, was considered to be medium. This assessment was subject to a high level of uncertainty associated with the true prevalence of *M. bovis* infection in fallen stock carcases in the HRA and the dose-response following ingestion of infected material in dogs. However, the fact that this is the first reported case in a kennel in the UK, may suggest that the actual risk is lower. On one hand this conclusion is caveated by the possibility of under-reporting through lack of systematic surveillance for TB in dogs. Alternatively, the conclusion is supported by a survey of culled hounds from ten Irish hunting kennels, over a three year period (2003-2005), which identified *M. bovis* infection in one of 52 foxhounds submitted for PME as part of the survey (Jahns, 2011).

As a proportionate risk mitigation strategy, DEFRA has introduced tighter restrictions on the collection and feeding of fallen stock to hounds in registered kennels. Since 10 October 2017 the feeding of offal from livestock species to dogs from recognised kennels or packs of hounds has been banned in England (Anon. 2017). Hunt kennel operators must also carry out additional examinations for lesions of TB in fallen stock originating from 'high risk' premises (defined as farms under movement restrictions due to a bovine TB breakdown, or which have been released from such restrictions in the previous 12 months). Additionally, APHA has developed training materials and guidance on the identification of TB in carcases of cattle and other livestock, for collectors and kennels feeding fallen stock to hounds. This should raise awareness of TB and assist recognition of tuberculous lesions in fallen stock for notification of suspicion of TB in carcases to APHA, as legally required under The Tuberculosis (England) Order 2014. The hunt kennel industry is also strengthening the voluntary code of practice and guidance for its members, to recommend PME of all hounds that die of unexplained or suspect causes.

5.6 Discussion

To the authors' knowledge this outbreak represents the only documented occurrence of tuberculosis in a canine species with evidence of onward dog-to-dog transmission within the affected group. Previous reports of tuberculosis in dogs have been of single sporadic cases or small case series of unrelated infections. The reason for, and source of, this outbreak remains unproven; however, given the known ability of *M. bovis* to spread readily between social animals, it is not surprising that a large group of kennelled hounds presented a pool of competent, susceptible hosts once the pathogen was introduced. Also remarkable in this outbreak was its fulminant nature, from the occurrence of the index case until the resolution of the outbreak (seven months), nearly one in ten animals on the premises became clinically sick. Tuberculosis is usually considered a chronic disease with only a small percentage of infected individuals becoming clinically unwell (Eisenhut, 2014). Therefore this rapid progression to disease in a relatively high proportion of animals can be considered unusual, although not unheard of, as sporadic fulminant human cases of renal tuberculosis leading to death by acute renal function impairment have been reported (Pathan, 2001; Dissanayake, 2004; Adzic-Vukicevic, 2017; Punia, 2008; da Silva Junior, 2016; Isao, 2006).

The reasons for the fulminant nature of this outbreak are likely to be multifactorial; a combination of pathogen virulence, host genetics and phenotype, as well as environmental conditions. There is a possibility that the pathogen became host-adapted. However, all animals from which viable organism was isolated were found to be infected with genotype 10:a. It may therefore also be the case that this strain of *M. bovis* in particular poses some genetic virulence with respect to the dog, or that it became tolerant to a new host and so spread amongst the hounds once introduced.

From a host perspective of transmission dynamics, the kennel stocking density was higher than that recommended by the Masters of Foxhounds Association (MFHA). This would have created an ideal opportunity for spread of disease once the index case became clinically sick and infectious. Similarly, the physiological stress of overcrowding may have made individuals

more susceptible to infection. A number of the kennels were in suboptimal states of repair. This meant that once the kennels became contaminated, it was almost impossible to appropriately disinfect them. They have since been destroyed completely and the kennel has moved to a new, clean site. Foxhounds *per se* are known to be at risk of mycobacterial infections with outbreaks of canine leproid granulomas reported in New Zealand and Australia (Smits, 2012). These outbreaks all affected closely related individuals and so the high level of genetic relatedness between individuals within this pack could imply that if a genetic susceptibility, such as a defect in cellular immune response did exist, then it would likely have high penetrance.

The majority of the clinical signs in sick hounds were non-specific and compatible with those seen due to *M. bovis* infections in other species; lethargy, anorexia, weight loss and lymphadenopathy (O'Halloran, 2017; Murray, 2015; Isaac, 1983). Unusually for companion animals, every clinically ill animal, those with lesions at PME as well as NVL hounds that were found to be culture positive, had renal infections. The kidney is not generally considered a so-called “target organ” for mycobacterial infections in companion animals, however, it is the most common site of extra-pulmonary TB in humans, other than lymph nodes (O'Halloran, 2017; Adzic-Vukicevic, 2017; da Silva Junior, 2016; Murray, 2015; Pesciaroli, 2014). Renal tuberculous infections are often considered to be insidious and present with mild or even subclinical disease, a significant proportion of cases are incidental findings at PME (Punia, 2008; Isao, 2006). In human cases of infection where irreversible kidney damage occurs, there are commonly co-morbidities. Amyloidosis is one such co-morbidity, particularly in India, which predisposes to glomerulonephritis and potentially fatal nephrotic syndrome (Sanz-Martín, 2016; Chong, 2017). This may also be the case for dogs; Foxhounds have previously been reported to be affected by renal amyloidosis (Mason, 1996), and this kennel has had previously confirmed clinical cases, whilst at least one of the *M. bovis* hounds was found to be positive for renal amyloidosis (data not shown).

Other conditions which adversely affect the outcome of renal tuberculosis are the deposition of anti-mycobacterial antibodies within the glomeruli and/or hypercalcaemia which is a

common complication of tuberculosis and is directly nephrotoxic (Zhao, 2017; Bellendir, 1978; Ko, 2004; Chan, 1994). It is possible that either of these conditions could have had an impact on the number of dogs affected and the severity of disease seen in this outbreak.

The relatively high level of renal immune-privilege may have allowed mycobacteria to replicate there whilst avoiding immune surveillance and clearance (Kurts, 2001). All of the hounds with renal lesions also had positive urine cultures (where these were available), which suggests that this could be a major source of infection for onward dog-to-dog transmission. However, if contaminated urine was the main source of infectious material, e.g. via aerosolisation, then lung pathology would be expected, but there were no pulmonary lesions in any of the dogs subjected to PME. Additionally, the urine cultures were only found to be positive after 14 weeks of culture suggesting that the initial number of organisms present was low. Based on the distribution of lesions it would appear that the mycobacteria arrived in the kidney by haematogenous spread. If so this indicates a distant source of infection which has not been identified.

Infected urine was identified as a key risk factor for potential human exposure during the risk assessment of kennel staff, predominately due to the use of power washers to clean excreta from the hard standing, which posed a significant risk of aerosolisation (Phipps *et al.*, manuscript submitted). The outcome of tuberculosis screening for at-risk humans at the kennels was that a single individual was diagnosed with latent tuberculosis (Phipps *et al.*, manuscript submitted). If urine from infected dogs was heavily contaminated with *M. bovis* it would have been likely that a higher proportion of individuals at risk would have been test positive. Based on these observational data, it is possible to conclude that *M. bovis* infected dogs are likely to be infectious to each other, and their urine may contaminate their local environment, but that this may not be the only or even the major route of transmission, and that the risk to human health is low.

No *ante mortem* tests have been validated for the diagnosis of *M. bovis* infection in dogs; we therefore combined two established testing methods for other species to try and maximise our

diagnostic sensitivity and bring this outbreak under control. The tests used here target the disparate arms of the immune response, with the IGRA evaluating the cell mediated Th-1 response whilst the serological assay used to detect a humoral Th-2 response. The percentage of tested animals deemed to be positive by each test was markedly different with 52% of dogs classed as infected by IGRA at initial screening but only 12.9% of dogs classed as infected or “at risk” by serology. This difference is likely due to the dominance of cell mediated immunity in an animals’ response to mycobacterial infection (Khan *et al.* 2006) but further investigation of the canine immune response to *M. bovis* and mycobacterial antigens is needed before this can be shown conclusively, this work is currently ongoing and will be published separately.

Whilst uncertainty exists with regards to the source of infection, it seems likely that contaminated fallen stock carcasses were involved. All culture positive animals had the same genotype isolated, which has not been frequently detected in the area local to the kennels. This suggests a single introduction with a large dose of infectious material was likely and that infection then spread between cohabiting hounds (Reynolds, 2006). One kennel worker was also diagnosed with latent tuberculosis, potentially due to exposure to infected hounds and/or their contaminated feed, though this remains unproven. Though the risk posed by *M. bovis* infected hounds to other hounds, cattle herds, local wildlife, their environment and their human keepers is low, this outbreak demonstrates that the risk is plausible and real. Resultantly, to try to prevent such an outbreak from occurring again, the conditions for the collection and use of animal by-products for feeding to dogs in recognised kennels or packs of hounds have now been amended to ban the feeding of offal from fallen stock. Similarly, health surveillance requirements for hounds have been increased by the MFHA. To be successful, these policy changes will need to be aided, however, by the future evaluation of diagnostic test accuracy and test development for canine tuberculosis.

5.7 References

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Chapter 6: Bovine Tuberculosis in Working Foxhounds: Lessons Learned from a Complex Public Health Investigation.

Preface

The following Chapter presents the findings of a study, conducted as part of this studenship programme that has been published in the journal *Epidemiology and Infection.*

The manuscript derived from this Chapter is available as an eponymous Open Access article: Phipps, E., McPhedran, K., Edwards, D., Russell, K., O'Connor, C., Gunn-Moore, D.A., O'Halloran, C., Roberts, T. and Morris, J. (2019). Bovine tuberculosis in working foxhounds: Lessons learned from a complex public health investigation. *Epidemiology and Infection,* **147,** E24.

Author contributions:

This public health investigation was devised and planned with input from the collective group of co-authors. The investigation was then implemented by Public Health England, supervised by Dr Morris and the manuscript prepared by Dr Phipps.

Abstract

In 2017, Public Health England South East health protection team were involved in the management of an outbreak of *Mycobacterium bovis* (the causative agent of bovine tuberculosis) in a pack of working foxhounds. This paper summarises the actions taken by the team in managing the public health aspects of the outbreak, and lessons learned to improve the management of future potential outbreaks.

A literature search was conducted to identify relevant publications on *M. bovis*. Clinical notes from the health protection database were reviewed and key points extracted. Animal and public health stakeholders involved in the management of the situation provided further evidence through unstructured interviews and personal communications.

The PHE South East team initially provided 'inform and advise' letters to human contacts whilst awaiting laboratory confirmation to identify the infectious agent. Once *M. bovis* had been confirmed in the hounds, an in-depth risk assessment was conducted, and contacts were stratified in to risk pools. Eleven out of twenty exposed persons with the greatest risk of exposure underwent tuberculosis screening and one tested positive, but had no evidence of active infection.

The number of human contacts working with foxhound packs can be large and varied. Health protection teams should undertake a comprehensive risk assessment of all potential routes of exposure, involve all other relevant stakeholders from an early stage, and undertake regular risk assessments. Current guidance should be revised to account for the unique risks to human health posed by exposure to infected working dogs.

6.1 Introduction

Detected infections of dogs with *Mycobacterium bovis*, the causative agent of bovine tuberculosis (bTB), are extremely rare, with only seven cases being reported to the Animal and Plant Health Agency (APHA) between 2004 and 2010 (1). The risk of transmission of *M. bovis* from dogs to humans is also considered rare, with expert consensus concluding that dogs are a 'spill over' host and not a significant source of transmission (2). However, this 2017 outbreak of *M. bovis* amongst a pack of working foxhounds in the South of England sparked considerable interest and concern from members of the public, media, veterinary and health professionals, and led to a co-ordinated response from Public Health England (PHE) health protection teams, the University of Edinburgh and the APHA (3; 4; 5; 6).

This was the first recorded outbreak of *M. bovis* in working foxhounds in England. Although public health guidance on the management of *M. bovis* associated with animals in general is available, there is no specific guidance describing the management of the human health risks associated with an outbreak of *M. bovis* in working foxhounds and the complex exposures this context presents. Given the increased awareness of *M. bovis* infection associated with working dogs (including foxhounds as seen here or other farm dogs) amongst the veterinary community, it is likely that assessments of the risks to human health will be required again in the future (1; 2). Such situations are likely to attract wider public interest due to concerns over the spread of bTB.

Management of this unique outbreak in working foxhounds required PHE to work collaboratively with veterinary stakeholders to identify exposed persons and limit the spread of *M. bovis* amongst both humans and animals. This paper summarises the actions taken by PHE South East in managing the public health aspects of this situation, and lessons that can be learned to improve the management of similar situations following notification of potential outbreaks of *M. bovis* in dogs or other working animals, especially in the non-household setting.

6.2 Methods

A literature search was conducted on PubMed and CAB Abstracts to identify relevant publications on *M. bovis* in dogs ((dog OR pack OR foxhound OR hound) AND (TB OR tuberculosis OR mycobacterium), all papers available on databases with English language translation available). Additional papers of relevance to this case study not identified through the literature search were suggested by the authors of the publication that arose from this work.

6.3 Results

6.3.1 Background epidemiology of *M. bovis* in the UK

Bovine tuberculosis is an important disease of cattle with a wide range of wild and domestic animal hosts (7; 8).The causative agent, *M. bovis,* is a slow growing, aerobic bacterium that typically causes infection of the lungs in humans, but can affect any organ in the body (9). Human infection with *M. bovis* typically occurs through ingestion, inhalation or contact with mucous membranes or skin abrasions of an infected animal (7). The main sources of zoonotic transmission of *M. bovis* infection are the ingestion of unpasteurised milk and dairy products, or prolonged exposure to aerosolised bacilli excreted from the respiratory tract of diseased animals (7; 10). Localised non-pulmonary *M. bovis* lesions can occur rarely through handling infected animals or carcases and theoretically through aerosolised exposure in abattoirs (10). Human to human transmission is extremely rare (9). In the United Kingdom, infection in humans with *M. bovis* is much less common than *M. tuberculosis*; between 2002 and 2014 there were 357 reported human cases of *M. bovis* compared to 96,887 cases of *M. tuberculosis* (9; 11). It is thought that most cases of *M. bovis* infection in humans in the UK are likely due to reactivation of latent infection acquired before compulsory pasteurisation of milk and bTB control programmes, contact with a human tuberculosis case, or infection acquired abroad (7; 9).

The incidence rate of *M. bovis* in cattle in England is one of the highest in Europe (11.0 new herd incidents per 100 herd-years at risk in 2017) (12). The UK government has implemented stringent controls to minimise the spread of bovine tuberculosis, with some counties in the South West and South East of England being deemed High Risk Area and separate adjacent buffer zone (Edge Area), and so cattle in these areas are subject to additional testing and surveillance (12). Non-bovine farmed animals such as South American camelids (alpacas), sheep, goats, pigs and deer are also sporadically infected, usually through direct or indirect contact with cattle or badgers (the two maintenance hosts of the bacterium in the UK) (13; 14). Infection of companion animals does occur, and there have been numerous reported cases of pets testing positive for *M. bovis* over the past few years, predominantly domestic cats (2; 12; 13; 15). The majority of cases of dogs infected with *M. bovis* in the literature come from countries other than England and have identified risk factors such as being strays or having exposure to infected non-domestic animals (1; 15; 16; 17).

The potential for zoonotic transmission of *M. bovis* from companion animals to human contacts was confirmed in 2014 when cases of human *M. bovis* infection followed transmission from infected cats in Berkshire, England (18; 19; 20). Lessons learned from the management of exposure to infected companion animals in 2014 justified a measured and co-ordinated public health response in order to protect human health.

6.3.2 Initial Risk Assessment

The responsible health protection team in the South East of England were initially made aware in early 2017 of the potential risk of zoonotic transmission of *M. bovis* by a private veterinarian with clinical suspicion of this diagnosis in working foxhounds cared for at the practice. A number of veterinary staff may have come into contact with infected hounds during their routine work. Key questions asked by the public health team in the initial assessment are summarised in Box 6.1, based on core principles of health protection practice and adaptation of existing general guidelines on the management of human health risks of tuberculosis (7; 21). Before implementing public health actions it is recommended to wait for laboratory

confirmation (7). Veterinary practice staff were therefore reassured and 'inform and advise' letters offered for distribution in the workplace, and the situation highlighted to the APHA in case of further action required.

Box 6.1: Initial Assessment

- What is the name of the hunt and where is it located
- How many animals are involved and how many have undergone testing
- What clinical signs did the hound(s) present with
- What type of testing has been undertaken (e.g. PCR/culture/IGRA)
- When are results available yet to confirm the diagnosis
- Any at risk groups in contact with the hounds e.g. immunocompromised staff
- Any symptomatic staff or household members?

Box 6.1: *Questions asked by the public health team in the initial assessment*

6.3.3 Confirming the Diagnosis

Primary concerns regarding the health of the kennel hounds were first raised in late 2016, a number of hounds had been euthanased for welfare grounds for reasons of deteriorating health within the preceding months. When a new hound starting showing similar clinical signs (weight loss, lethaergy, hyporexia, polyuria and polydipsia) it was euthanased and submitted for *post-mortem* examination. Gross renal pathology was confirmed histopathologically as granulomatous, and the presence of acid-fast bacilli with mycobacterial morphology was confirmed by examination of Zeihl-Neelsen stained sections of diseased tissue.

This finding instigated a veterinary disease outbreak investigation by the Univeristy of Edinburgh, Biobest Laboratories and APHA (detailed in reference 24). Briefly, 164 hounds in the kennel were tested using an experimental interferon-gamma release assay (IGRA) test at Biobest Laboratories and a serological assay originally developed for cervid species (Dual Path Platform VetTB test for cervids).

Of the 164 hounds tested, 85 (52%) were diagnosed as being test positive *as per* the prospective case definition set. Test positive hounds and clinically sick hounds were euthanased and *M. bovis* infection was confirmed by culture in 14 cases. The isolated

organism was genetically tested confirming genotype 10:a with the first laboratory confirmation occurring in February 2017.

6.3.4 Veterinary Epidemiological Investigation

Once infection with *M. bovis* 10a had been confirmed evaluation of the risk to human health was undertaken (see below). Simultaneously, an epidemiological investigation began to identify the risk pathways by which the hounds may have initially become infected. These are detailed in (24) and comprised of, in order of considered likelihood; a) movement of infected hounds into the kennels b) feeding *M. bovis* infected fallen stock to the hounds c) exposure to infected livestock or wildlife during work and/or d) exposure to infected local wildlife at the kennels.

Qualitative assessment of each pathway was undertaken with the evidence available. Tracing of hound movements indicated that 21 had been moved onto the affected premises within the three years prior to the diagnosis of the index case, of which 18 were moved in the preceding 18 months. Some kennels of origin of these hounds were located within the geographical region of the UK designated as the High Risk Area (HRA) for *M. bovis* incidence with respect to bovine infections. Furthermore, a number of these were located within the home-range of *M. bovis* 10a. It was therefore considered to be medium risk (and therefore most probable) that whilst at these kennels, hounds were fed fallen stock infected with *M. bovis* 10a and this was the source of infection.

Due to the outbreak-kennel location within the Edge Area for *M. bovis* incidence, being out with the home range of *M. bovis* 10a and with no locally identified wildlife infections with *M. bovis* the remaining pathways were deemed to be possible sources of infection but low risk.

6.3.5 Identifying At Risk Groups

Following culture confirmation of *M. bovis* from the hound tissue samples, PHE undertook a formal risk assessment to identify potential routes of zoonotic transmission, understand levels of exposure and determine future public health actions, including possible screening of human

contacts. Available guidance related to *M. bovis* in livestock was reviewed and tailored to this specific situation (7). The key points of this risk assessment are summarised in Box 6.2, and involved collecting information from staff caring for the foxhounds, the local veterinary practice and the APHA. Again, this risk assessment was undertaken based on key principles of health protection practice and adaptation of general guidance on the management of human health risks related to bTB (7; 21).

Box 6.2: Summary Details of the Public Health Risk Assessment

Potential routes of exposure

- Identify all persons who were in contact with the symptomatic foxhounds including temporary and previous staff members at the kennels and veterinary practice
- Define the level of contact and activities undertaken e.g. preparing food, grooming, cleaning environment, dressing wounds, undertaking invasive procedures
- Whether anyone may have been bitten by infected foxhounds

Risk of exposure

- Level of exposure – total time spent with infected foxhounds by each person involved
- Use of personal protective equipment (PPE) during contact – consistency and type worn
- Whether any contact could have generated aerosols

Environmental factors

- Environments in which contact takes place e.g. kennels, household, vehicles and veterinary practice
- Condition of kennels e.g. cracked concrete and other porous surfaces
- Ventilation and cleanliness of environments
- Frequency and level of cleaning undertaken e.g. sweeping, pressure washing, disinfecting

Veterinary assessment

- List of veterinary practices caring for the working foxhound pack
- Prevalence of bTB in the surrounding area amongst bovine and non-bovine animals
- Potential route of initial infection e.g. contact with confirmed bovine cases or being fed potentially contaminated meat
- Follow up being undertaken by APHA
- Any plans to test the rest of the working foxhound pack

Box 6.2: *Outline of the agreed basis for HPE risk assessment*

6.3.6 Communications Strategy

A multi-agency incident control team (ICT) was convened to facilitate effective information gathering and to agree on suitable actions for all involved stakeholders. This collective

approach, also involving specialist communication colleagues from all agencies, ensured that consistent messages were delivered, with the aim of reducing misinformation and managing perceptions of risk amongst the public (22).

The foxhounds were exercised on land and at events over several counties in the South East. Local health protection teams (HPTs) across the region received calls from members of the public, veterinary and public health professionals enquiring about the pack and associated risks of infection. Calls to HPTs were recorded on a case and incident management database HPZone (inFact Shipley, 2012). In order to facilitate co-ordination between teams and quick referencing, a unique identifier for the situation was created that could be linked to all incoming enquiries taken by the teams involved. The response was coordinated across several HPTs by the incident lead, and an email circulation list was used to keep the incident management team informed of all actions undertaken and updates on the situation. PHE and DEFRA communications teams were also involved at this point to agree on what proactive and reactive statements would be appropriate. DEFRA and veterinary colleagues experienced a higher volume of interview and statement requests than the public health teams, likely due to the high profile of fox hunting and bTB in the media.

6.3.7 Screening & Further Public Health Actions

A ‘stone in the pond’ approach was adopted in determining which human individuals should initially undergo screening for tuberculosis (23). Screening does not differentiate between *M. bovis* and *M. tuberculosis* infections, but does identify persons requiring further investigations and confirmatory testing. PHE identified close contacts with the highest risk of potential transmission for initial screening, and then planned to expand the screening pool to more casual contacts if the first round of screening suggested significant transmission had occurred.

The individuals identified for the first round of screening were those with the greatest degree of contact with infected foxhounds, and included persons who had conducted invasive procedures on symptomatic dogs (such as *post-mortem* examinations and surgical procedures) without appropriate personal protective equipment (PPE), and kennel workers

involved with food preparation and cleaning of kennels using a pressure washer. These were hypothesised to be the highest risk exposures to potential sources of *M. bovis*, e.g. infected tissue, aerosolised fluids and potential contaminated fallen stock used as feed. The use of a pressure washer was of particular interest as the presence of kidney lesions in infected foxhounds (24) suggests that *M. bovis* bacilli could have been excreted in the hounds' urine.

Persons in the initial screening group were referred to their local tuberculosis service for screening. All other identified potential contacts were sent an 'inform and advise' letter whilst results from the initial round of screening were awaited (see Box 6.3 for outline summary). In addition, HPT staff visited the veterinary practice to discuss and allay staff concerns related to *M. bovis* and their risk of acquiring tuberculosis.

Box 6.3: Key information for inform and advise letters

- Reassure that the risk of transmission from animals to humans is low
- Some persons may be unusually susceptible such as immunocompromised persons
- Description of the symptoms of tuberculosis
- Advice to contact GP if experiencing any of these symptoms, mentioning the possible route of exposure through the hunting pack
- Screening of high risk persons is currently taking place and we may be in contact again to arrange screening if initial tests indicate that transmission of bTB has occurred

Box 6.3: Agreed purpose of communication strategy with at risk individuals

In total, eleven out of seventeen people were considered to have potential exposure to infected foxhounds as outlined in Figure 6.1 and were offered tuberculosis screening. Of these, one person tested positive for TB (using QuantiFERON™ TB Gold). Following a further assessment including computed tomography (CT) scanning and culture of tissue samples, there was no evidence of active tuberculosis infection and latent tuberculosis was diagnosed. This person had not had previous testing for tuberculosis, did not have other risk factors, but was involved in all high risk activities including *post-mortem* examination without PPE, preparation of fallen carcases for feeding, cleaning of kennels using a pressure washer and care of open wounds on infected animals. Seven contacts screened negative for tuberculosis,

and three declined the offer of screening. Based on this outcome of the first screening round, it was agreed by the ICT that further screening would not be offered to more casual contacts.

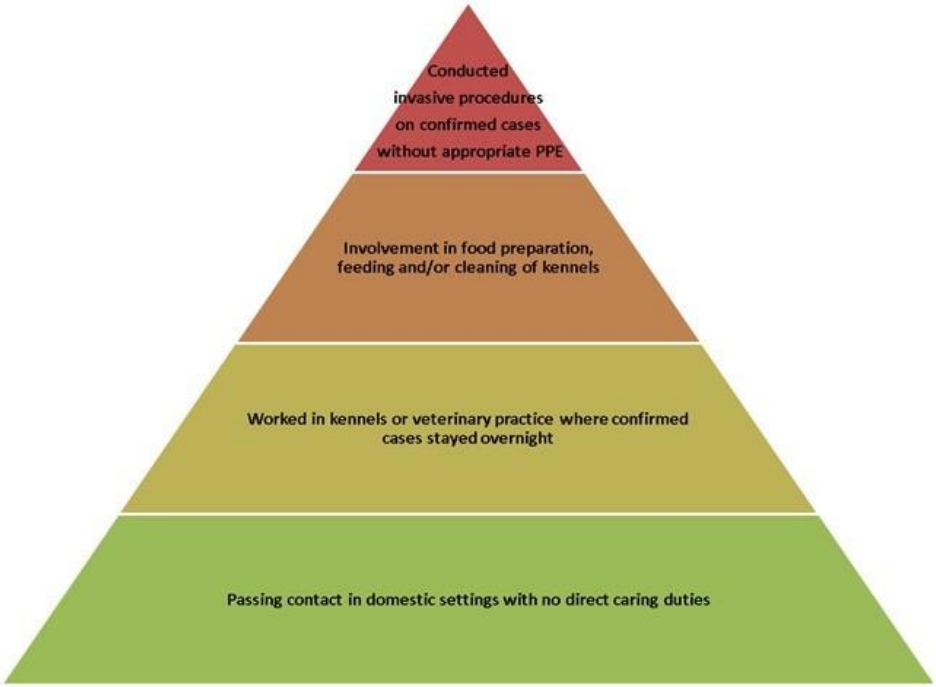

Figure 6.1 Hierarchy of screening pools used during the public health investigation resulting from the kennel outbreak of *Mycobacterium bovis* tuberculosis in working foxhounds.

Screening of the foxhounds continued over several months with two further screening rounds; a total of 164 hounds were tested with a 52% IGRA positivity rate (24). All foxhounds testing IGRA positive were euthanised. A second meeting of the original multi-stakeholder ICT was convened to discuss how to manage the ongoing exposure to IGRA positive hounds since the initial diagnosis. Staff members who had declined screening initially were encouraged to attend given this ongoing exposure. Three new staff members (bringing the total number of screened persons up to 20) who had joined the hunt since the initial diagnosis were identified and they were provided with 'inform and advise' letters to highlight their potential risk of exposure to infected foxhounds during both rounds of animal screening, and recommended actions if they develop symptoms indicative of potential tuberculosis infection.

6.4 Discussion

The management of this situation has highlighted several learning points for veterinary and public health stakeholders. Outbreaks of potentially zoonotic infections amongst working foxhound packs are a unique challenge for both health protection teams and veterinary investigation teams, as they are comprised of large populations (in this case over 150) individuals, which are highly mobile (this kennel covered an area spanning six counties) and hounds are legislatively defined neither as companion animals nor agricultural livestock, meaning for example, that they can be fed fallen stock. The hounds from working packs may span both categories, spending time in both domestic and farming settings. This makes quantifying the unique exposures and risk of transmission associated with this context particularly difficult as they are not specifically covered by any existing guidelines. Moreover, IGRA testing is not validated for use in diagnosing *M. bovis* infections dogs, so the sensitivity and specificity of any results are unknown, adding some uncertainty to whether public health action should be warranted in light of a positive test (25). In this scenario, given the high number of positive IGRA tests, confirmatory tissue cultures and foxhounds with clinical signs consistent with tuberculosis, the decision to take action was relatively straightforward, but could be difficult in the face of less conclusive results.

Bovine tuberculosis is an understandably emotive subject for many stakeholders, including kennel staff, farmers, veterinarians and the general public, and there was considerable media interest. It was crucial to ensure that confidentiality of the working foxhound pack was protected as much as possible by agencies involved, and that information sharing activities were coordinated and agreed on by all parties. The geographical area covered by the pack during outings spanned several counties, and required the involvement of multiple HPTs and tuberculosis services to coordinate a response. Effective communication between these teams is extremely important to ensure that resources are used efficiently and messages are consistent in order to address public concern. By visiting the veterinary practice in person, the health protection team were able to build relationships and foster trust from stakeholders to manage concern successfully. This also presented an opportunity for highlighting the

importance of appropriate wearing appropriate personal protective equipment when performing *post-mortem* examinations of animals, which would have significantly reduced the risk of exposure to *M. bovis* to veterinary and kennel staff.

This situation highlighted how dynamic risk assessments should be undertaken frequently during the ongoing process of outbreak management, as the foxhound pack is likely to come in to contact with new persons such as temporary staff and members of the public as well as those potential contacts identified in the initial assessment. IGRA testing of the whole pack was a significant undertaking for the kennel and University of Edinburgh staff, who were unable to secure additional funding for testing as the foxhounds were deemed to be non-livestock species, and took several months to complete. During this period, workers continued to be exposed to hounds that were eventually found to be IGRA positive. Managing this unknown and unquantifiable risk of potential exposure whilst waiting for IGRA results was particularly challenging, and involved concerted effort from the HPT to communicate effectively with stakeholders to address and allay concerns. Senior managers of the foxhound kennels were very receptive to both public health requirements and advice regarding animal health management. It is worth noting that as the hounds are not classed as livestock, the APHA have no regulatory powers to enforce euthanasia or other control methods.

6.5 Conclusion

This paper highlights the unique and unusual health protection scenario of managing potential working foxhound to human transmission of *M. bovis*. The number of human contacts with working packs can be large and varied, and the animals are not considered as domestic companion animals or livestock. Health protection teams involved in the management of such situations must ensure to undertake a comprehensive risk assessment of all potential routes of exposure, involve all other relevant stakeholders from an early stage in developing management and communication plans, and undertake regular risk assessments as new information becomes available. Current guidance should be revised to account for the unique risks to human health posed by exposure to infected working dogs.

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Chapter 7: Comparison of Potential *Ante Mortem* Assays for the Identification of *Mycobacterium bovis*-Infected Domestic Dogs (*Canis lupus familiaris*).

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Abstract

The domestic dog (*Canis lupus familiaris*) is a competent host for *Mycobacterium (M.) bovis* infection but no *ante-mortem* diagnostic tests have been validated for this species. The aim of this study was to compare the performance of potential *ante-mortem* diagnostic tests across samples collected from dogs considered to be at high or low risk of sub-clinical *M. bovis* infection. We tested 164 dogs at high risk of infection and 119 dogs at low risk of infection with a combination of cell-based and/or serological diagnostic assays previously described for use in non-canid species. The interferon-gamma release assay (IGRA) using peripheral blood mononuclear cells (PBMC) identified the highest number of test positive animals (85, 52%), with a suggested specificity of 97.3%, whilst a whole blood IGRA was found to be unreliable. The production of antigen-specific tumour necrosis factor-alpha (TNF-α) by PBMC in response to a cocktail of ESAT-6 and CFP-10 antigens correlated very strongly with the overall IGRA

results and has future diagnostic potential. All three serological assays employed in this study (Idexx *M. bovis* Ab ELISA, [Idexx Laboratories, USA], DPP VetTB lateral flow assay [Chembio, USA] and comparative PPD ELISA [in-house]) identified seropositive dogs but, overall, the test-positive rate for the serological assays was only one third that of the cellular based assays. Circulating serum cytokine concentrations of interferon gamma (IFN-γ) and TNF-α were not statistically different between high and low risk groups of dogs. While many dogs in the high risk group had serum biochemical abnormalities, these did not correlate with the findings of other diagnostic tests.

This study demonstrates, for the first time, the potential utility of two cellular and three serological assays for detecting sub-clinical *M. bovis* infections of dogs. Whilst the data suggest high test specificity for all assays evaluated, further work is needed to validate the sensitivity and specificity of individual or combinations of tests using sufficient numbers of dogs of known infection status.

7.1 Introduction

The domestic dog (*Canis lupus familiaris*) is, like many mammalian species, a competent host for *Mycobacterium (M.) bovis* infection (Snider *et al.* 1971; Ellis *et al.* 2006; Broughan *et al.* 2013a). Although clinical cases of tuberculosis (TB) due to infection with *M. bovis,* or other members of the *M. tuberculosis*-complex (MTBC), are considered rare in the dog and generally only occur sporadically (Broughan *et al.* 2013b), we previously investigated a fulminant disease outbreak caused by *M. bovis* infection within a large group of kennel-housed working Foxhounds in the UK (O'Halloran *et al.* 2018). Investigation of this outbreak was impeded in its speed, and by the lack of available *ante-mortem* diagnostic tests for identifying *M. bovis* infections in domestic dogs (Snider *et al.* 1971; Snider *et al.* 1975; Broughan *et al.* 2013b). The tests employed therefore had to be adapted and interpreted in real time, based on extrapolation of performance data from non-canine species.

Interferon gamma (IFN-γ) release assay (IGRA) tests have been developed on the principle of quantitatively evaluating antigen-specific IFN-γ production by peripheral circulating effector

memory T-cells following *in vitro* stimulation (Adams 2001; de la Rua Domenach *et al.* 2006; Schiller *et al.* 2009). The first IGRA was designed to increase the sensitivity of *M. bovis* TB testing in cattle (Wood *et al.* 1990), and the resulting BOVIGAM™ assay holds World Organisation for Animal Health (OIE) validation, with a reported in-field sensitivity of 81.8-100% and specificity of 88-99% (Wood, 2001; Vordermeier et al., 2006; Schiller et al., 2009; Bezos, 2014). IGRA tests have subsequently been adapted to identify active and latent TB in human patients with at least equivalent sensitivity and increased specificity when compared to the intradermal tuberculin skin test (TST) (Kim, 2011; Zhou, 2011; Eisenhut, 2014; Thillai, 2014). Similarly, IGRA test protocols have been developed for use in domestic cats with a suggested high sensitivity and specificity for the detection of MTBC infections, and for the detection of *M. tuberculosis* infections in dogs in a high risk setting (Rhodes *et al.* 2011; Parsons *et al.* 2012; Broughan *et al.* 2013b); while the same study found the intradermal TST to be unreliable in the dog (Parsons *et al.* 2012).

The IGRA used in this study was specifically developed to test dogs for subclinical *M. bovis* infection, and was based on the protocol used for testing domestic cats (Rhodes *et al.* 2011); the antigens purified protein derived (PPD) from *M. avium* (PPDA), PPD from *M. bovis* (PPDB) and a cocktail of proteins derived from the immunodominant proteins; 6kDa early-secreted antigenic target (ESAT-6) and 10kDa culture filtrate protein (CFP-10) were selected. PPDA is frequently used with PPDB as a comparator in both IGRA and TST tests to assess and mitigate for exposure/sensitisation to, or infection with, environmental mycobacteria species. Infection with a MTBC mycobacteria is confirmed if the IFN-γ response of an animal is greater to PPDB than to PPDA (Wood, 2001; Vordermeier *et al.* 2007).

The proteins ESAT-6 and CFP-10 are both encoded on the RD-1 region of most MTBC, including *M. bovis*, and a small number of non-tuberculous mycobacteria (NTM) (Harboe *et al.* 1996; Brosch *et al.* 2002). They are secreted proteins that form a heterodimeric complex in a 1:1 ratio which has been consistently associated with phagocyte lysis and pathogen virulence (Gao *et al.* 2004; Guinn *et al.* 2004; Junqueira-Kipins *et al.* 2006). The presence of a concurrent response to the recombinant combination of ESAT-6/CFP-10, as well as PPDB,

indicates infection with an RD-1 positive MTBC mycobacteria (*i.e.* it excludes infection with *M. microti* or previous vaccination with *M. bovis*-BCG) (Harboe *et al.* 1996; Buddle *et al.* 2001; Brosch *et al.* 2002).

A number of studies have tried to improve the sensitivity and/or specificity of IGRA tests. The overall performance of an IGRA, when used to test cattle for *M. bovis* or humans for *M. tuberculosis* infection, can be increased by the measurement of additional cytokines secreted by antigen specific cells within the blood (Jones; 2010; Elnagger *et al.* 2017). This includes tumour necrosis factor-alpha (TNF-α), which has a well described role in the mammalian host immune response to mycobacteria (Allie *et al.* 2013, Leem *et al.* 2018).

The bovine (BOVIGAM™) and human (QuantiFERON™ TB Gold) IGRA assays are both performed on whole blood without first needing to isolate peripheral blood mononuclear cells (PBMC), as is required for IGRA testing cats, camelids and the T-SPOT.*TB*™ test (Hesketh *et al.* 1994; Gutierrez, *et al.* 1998; Vordermeier *et al.* 2007; Rhodes *et al.* 2008a*;* Igari *et al.* 2018; Leem *et al.* 2018). The use of whole blood rather than PBMC makes the former assays quicker and cheaper to conduct and so would be an ideal development for canine TB testing.

Both IGRA and TST assays evaluate the cell mediated adaptive immune response (CMI) of an animal which dominates the reaction to intracellular pathogens, including mycobacteria (Allie *et al.* 2013). However, skin test responses in dogs have been shown to be unreliable (Parsons *et al.* 2012). Diagnostic serological assays, while generally recognised as having lower sensitivity than CMI assays, have been used both experimentally and commercially for the diagnosis of MTBC infections in a number of species (Min *et al.* 2011; Rhodes *et al.*, 2012 El-Seedy *et al.* 2013; Casal *et al.* 2014; Roos *et al.* 2016; Waters *et al.*, 2017, Chambers *et al.*, 2010). It is recognised that combining serological and CMI diagnostic tests may provide added value by increasing infection detection, e.g. Bezos *et al.* (2018) showed an increase in overall diagnostic sensitivity for both cattle and goats with MTBC infections.

M. bovis is an endemic pathogen of major significance to the UK farming industry as the causative agent of bovine tuberculosis (bTB) (Vordermeier *et al.* 2007). In England and Wlaes

the regions with high prevalence of *M. bovis,* as defined by the incidence of bovine infections, comprise the High Risk Area (HRA), and lower but still significant disease prevalence occurs within counties of the Edge Area. Control measures implemented for cattle infections amount to an estimated cost of £100 million annually, and while the most recent government statistics show a decrease in herd incidence in England, there is a mixed picture, with an increase in the number of cattle slaughtered annually (DEFRA, 2018).

Non-bovine reservoirs of infection are increasingly being investigated for their potential role in the epidemiology of the disease; either as possible reservoirs of infection or as sentinel species, but species-specific diagnostic tests are often lacking which impedes such investigations (Buddle *et al.* 2000; Delahay *et al.* 2002). As the UK moves towards its goal of *M. bovis* elimination, having diagnostic tests with known accuracy for such spill over species will be critical.

The aim of this study is to compare the performance of *ante-mortem* diagnostic tests across samples collected from individual dogs considered to be at either high or low risk of sub-clinical *M. bovis* infection, and (presumed) TB-free dogs.

7.2 Materials and Methods

7.2.1 Sample collection

Diagnostic blood samples from animals considered to be at high risk of sub-clinical infection with *M. bovis* (high risk group, HRG) were those taken from 164 kennel-housed foxhounds during an outbreak of TB due to *M. bovis* which occurred between December 2016 and July 2017 (Table 7.1) (O'Halloran *et al.* 2018). Whole blood and PBMC stimulation assays were conducted at the time of sampling, whilst serological assays were performed subsequently on aliquots of serum separated and frozen at -80ºC within 24 hours of collection.

To allow for a comparison of test performance in populations with predicted differences of infection prevalence, we also tested a total of 119 opportunistically collected blood samples

(*i.e.* remnant blood from samples taken for clinical reasons unrelated to this project); 77 from presumed TB-free dogs (TB-free group) and 42 dogs with some degree of potential exposure to cats or dogs with a mycobacterial infection (low risk group, LRG). The TB-free group provided 45 serum samples and 18 PBMC samples, an additional 42 PBMC samples were available from the LRG dogs, as shown in Table 7.1.

Table 7.1: Origin of blood samples used in this study: *serum was unavailable for one Foxhound, HfSA; Hospital for Small Animals; **All samples from dogs resident in Scotland which has held Officially TB Free (OTF) Status since 2009.

High risk or Low risk groups	**Group of dogs**	**Total number**	**Number of serum samples obtained**	**Number of PBMC samples obtained**
High risk	Kennel Foxhounds where the *M. bovis* outbreak occurred	164	163*	164
TB-free	Canine patients at the HfSA, University of Edinburgh**	45	45	0
TB-free	Blood donor dogs at the HfSA, University of Edinburgh**	24	0	24
TB-free	Canine geriatric health screen patients, HfSA University of Edinburgh**	8	0	8
Low risk	Pet dogs in contact with a household (animal) case of mycobacterial disease	10	0	10
Low risk	Pet dogs of kennel staff where a previous *M. bovis* outbreak occurred	32	0	32

Remnant samples were only included in this study if, prior to blood sample collection, the dog had not been treated with immunomodulatory medications e.g. non-steroidal anti-inflammatory drugs (NSAIDs), chemotherapeutic agents or corticosteroids within 14 days prior to sample collection. Dogs were excluded if they were pre-treated with antibiotics with efficacy against mycobacteria, including fluoroquinolones, macrolides/azides or doxycycline within the same 14 day period. Dogs were not excluded if they had been treated with antimicrobial agents if these would be ineffective against mycobacteria, such as a penicillin or cephalosporin. Similarly, dogs were not excluded if they had been treated with non-immunomodulatory analgesic medications e.g. opioids.

This study was conducted following approval from the School of Veterinary Medicine Ethical Review Committee at the University of Edinburgh; all relevant guidelines and regulations were adhered to throughout.

7.2.2 IGRA using isolated PBMC

The IGRA assays for the 164 dogs in the HRG were conducted as previously reported (O'Halloran *et al.* 2018). The same assay was used to test PBMC isolated from the 74 dogs in the TB-free and LRG groups (Table 7.1). The assay protocol had been validated and previously published for use in the domestic cat and was modified for use in the dog (Rhodes *et al.* 2011). A sample of up to 5ml heparinised whole blood was taken from each animal and transported to the laboratory at ambient temperature within 18 hours. Upon receipt, blood was diluted 1:1 with Hanks Balanced Salt Solution (HBSS, Gibco, UK) and layered over Histopaque 1077 (Sigma, UK) before centrifugation at 800 x *g* for 40 minutes at room temperature. The PBMC were removed from the resulting interface, washed with HBSS and re-suspended in complete culture media (RPMI 1640 containing 100µg/ml L-glutamine, 10% foetal bovine serum, 100µg/ml penicillin, 100U/ml streptomycin, $5x10^{-5}$M 2-mercaptoethanol and non-essential amino acids) to a density of $2x10^{6}$/ml. A 100µl sample of the PBMC suspension was stimulated in duplicate with PPDA or PPDB (Lelystad, Prionics, Netherlands); both at a final concentration of 10µL, as well as a peptide cocktail of ESAT-6/CFP-10 at a final concentration

of 5ug/ml (Lionex, Germany), a mitogen positive IFN-γ control of phorbol myristate acetate plus calcium ionophore (PMA/Ca, Sigma, UK, 50ng/ml and 1µg/ml respectively), and finally a complete culture medium only (i.e. negative) control.

Cells were incubated for four days at 37°C/5% CO_2, after which the supernatants were removed for quantification of IFN-γ by ELISA. Supernatants were either directly assayed or stored at -80°C until required. The IFN-γ ELISA was conducted using a commercially available canine specific ELISA kit (DY781B, R&D Systems, Europe Ltd., UK) according to the manufacturer's instructions.

Supernatant from each cell culture condition was assayed in duplicate. Optical density (OD) values were measured at a wavelength of 450nm and 630nm; the replicate OD (450nm-630nm) values for each condition were averaged to give the final OD values and standard deviations were calculated. Where replicates differed by more than 30% from the mean, the test was considered invalid.

During the outbreak, a prospective case definition was set to determine which hounds would be considered IGRA test positive (O'Halloran *et al.* 2018). It was determined that a statistically significant response to any of the three test antigens (PPDB, PPDA or ESAT6/CFP10) above the negative condition (media control) was indicative of a biologically significant T-cell response. The responsiveness threshold for each dog was defined as the mean OD value of the media negative control plus two standard deviations (2SD). The mean antigen-specific response minus two standard deviations must exceed this for response to be considered positive test (*i.e.* there must be non-overlapping 2SD). Similarly a PPDB-biased response was one where the mean PPDB response minus 2SD was greater than the mean PPDA response plus 2SD. For the test to be considered valid, the PMA/Ca (sample positive control) response must also be positive by the same criteria when compared to the negative sample control. As many of the PBMC positive control and antigen specific response values were above the linear range of the test kit standard curve, the OD values were used for test result interpretation.

7.2.3 TNF-α assay

The concentration of TNF-α was measured in PBMC IGRA supernatants. Cell supernatants from a randomly selected subset of 45 dogs from the HRG were assayed by commercial ELISA (DY1507, R&D Systems, Europe Ltd., UK) according to the manufacturer's instructions. Since PMA/Ca stimulation, as used as a positive control for the production of IFN-γ from lymphocytes in the IGRA, did not induce TNF-α in any dog sample tested, and antigen-specific TNF-α responses were observed, Concanavalin A (ConA) was added to the positive control mitogen mix in a supporting experiment using three healthy dogs (from the TB-free blood donor subset, Table 7.1). The PBMC were isolated and incubated with PMA/Ca in the same conditions as described above but with the addition of ConA at a final concentration of 25µg/ml. After incubation for four days at 37ºC/5% CO_2, the culture supernatant was assayed for TNF-α and IFN-γ by ELISA following the manufacturer's instructions.

Samples from the selected 45 dogs were interpreted as positive where the mean antigen-specific TNF-α response minus 2SD was greater than the mean media sample negative control plus 2SD. Similarly a PPDB-biased response was one where the mean PPDB response minus 2SD was greater than the mean PPDA response plus 2SD.

7.2.4 IGRA using antigen stimulated whole blood

To compare the diagnostic results of whole blood and PBMC stimulation assays, a whole blood assay was conducted on the same subset of 45 dogs in the HRG tested in Section 7.2.3. Whole blood stimulation was performed using heparinised whole blood. One millilitre of heparinised whole blood was added to each of five separate wells of a 12-well tissue culture plate (ThermoFisher Scientific, USA) to which the following had been added; (a) 25µL phosphate-buffered saline (PBS, pH 7.2) (b) 25µL PMA/Ca (Sigma, UK) to a final concentration of 50ng/ml and 1µg/ml respectively (c) PPDA or PPDB, to a final concentration of 10µg/mL (Lelystad, Prionics, Netherlands), (d) 25µL of purified ESAT-6 to a final concentration of 5µg/ml and (e) 25µL of combined ESAT-6/CFP-10, to a final concentration of 5µg/ml each. The plates were incubated overnight (approximately 16 hours) at 37ºC/5% CO_2.

The following day, the plates were centrifuged at 800 x *g* for 15 minutes and the plasma was removed from the cellular fraction before being stored frozen at -80ºC. The concentration of IFN-γ in the samples was subsequently determined by ELISA (DY781B, R&D Systems, Europe Ltd., UK). Plasma samples from each condition were assayed in duplicate. The mean replicate OD (450-630nm) values were used to determine the concentration of IFN-γ against a recombinant canine IFN-γ standard curve (R&D Systems) and values are reported in pg/ml.

7.2.5 Serological testing with commercial tests; the DPP VetTB Assay and Idexx *M. bovis* antibody ELISA

Sera from each of the 163 HRG dogs and the 45 sera from TB-free dogs were tested by Dr Shelley Rhodes (APHA) using the DPP VetTB test (Chembio Diagnostic Systems, Inc., Medfords, New York, USA) as previously reported (O'Halloran *et al.* 2018). Briefly 30μl of serum was applied to a single cassette and washed across two antigen lines (MPB83 and ESAT-6/CFP-10) with the kit buffer. After five minutes a further buffer application was applied *as per* the manufacturer's instructions to wash the colloidal gold detecting reagent across the antigen and test control lines. After 15 minutes each cassette was visually inspected for QC purposes (to confirm the presence of a visible positive control line) and the antigen-specific antibody binding was quantified by inserting the cassette into an optical reader (Optricon DPP Reader, Chembio) which measures the reflectance of the response produced to both antigens, MPB83 and ESAT-6/CFP-10, individually as relative light units (RLU).

Sera from the same animals (HRG and TB-free) were also tested by Dr Rhodes using the Idexx *M. bovis* antibody (Ab) ELISA (Idexx Laboratories, Inc., Westbrook, ME), performed with minor modifications to detect canine antibodies. ELISA plates pre-coated with mycobacterial antigens (MPB83 and MPB70) were supplied by Idexx, together with positive and negative controls and a secondary antibody to bovine IgG antibody. The kit secondary antibody, while retained for the kit-positive and -negative bovine plate controls, was replaced with Protein-G conjugated to horseradish peroxidase (HRP) which binds the constant region of antibodies of numerous species. Serum samples were diluted 1:50 and added to the ELISA plate in

duplicate, together with plate positive and negative controls, and ELISA plates were incubated for one hour at room temperature. Plates were washed with wash buffer (supplied with the kit), and then incubated with Protein-G-HRP for 30 minutes at room temperature and then washed again. Substrate (supplied with kit) was added to each well (100µL per well), the plates were developed for 15 minutes at room temperature, and the reaction was stopped by adding 100µL per well of stop buffer (supplied with kit). Absorbance values were read at 450nm *as per* the manufacturer's instructions and the mean of each sample replicate calculated as a final OD value test result.

The HRG and TB-free group results were compared by Mann Whitney U test with a P value of less than 0.05 considered to be significant.

7.2.6 Serological testing by in-house comparative PPD ELISA

ELISA test plates (NUNC Maxisorp Immmunoplate F96, Scientific Laboratory Supplies Ltd., UK) were coated with 100µL of test antigen (PPDA or PPDB) diluted to 10µg/mL in carbonate-bicarbonate buffer (pH 9.6, Sigma Aldrich, UK). Plates were sealed and incubated at room temperature (RT) overnight. Plates were washed with PBS containing 0.05% Tween 20 and were blocked for one hour at RT with blocking buffer comprised of 4% bovine serum albumin (BSA; Sigma Aldrich, UK) in PBS. Serum from each dog (HRG, n=163 and TB-free, n=45) was diluted 1:50 in 2% BSA in PBS. After the plates were washed, 50µL of diluted serum was added to test wells in duplicate, blocking buffer was added to two wells on each plate (blank control). Plates were sealed and incubated at RT for two hours. Plates were washed again and 50µL of detection antibody (HRP-conjugated goat-anti-canine IgG; Euroimmun, Lübeck, Germnany) was added to each well and incubated for 45 minutes at RT. Following incubation, plates were again washed and 50µL of 3,3',5,5'-tetramethylbenzidine (TMB; Sigma Aldrich, UK) added. After a 15 minute incubation, 25µL of 2M H_2SO_4 stop solution was added.

Absorbance was read at 450nm and 630nm, test results for each dog were calculated as mean OD (450-630nm) of replicate wells with the mean OD (450-630nm) of blank control wells from

that plate subtracted. For the comparative test, the final OD value for PPDA was subtracted from the value for PPDB, giving an overall PPD OD value.

The HRG and TB-free group results were compared using Mann Whitney U test with a P value of less than 0.05 considered to be significant.

7.2.7 Serum IFN-γ and TNF-α ELISA

Serum from sampled dogs (HRG, n=163 and LRG, n=45) was assayed for IFN-γ and TNF-α by commercial ELISA as described above (Sections 8.2.2 and 8.2.3); 50µL of serum diluted 1:10 in PBS was assayed in duplicate for each dog. The calculated OD value (mean of the replicate OD (450-630nm) values) was used to determine the concentration of IFN-γ or TNF-α respectively, against the standard curves. Values were calculated in pg/mL; the results were statistically compared by Mann Whitney U test with a P value of less than 0.05 considered to be significant.

7.3 Results

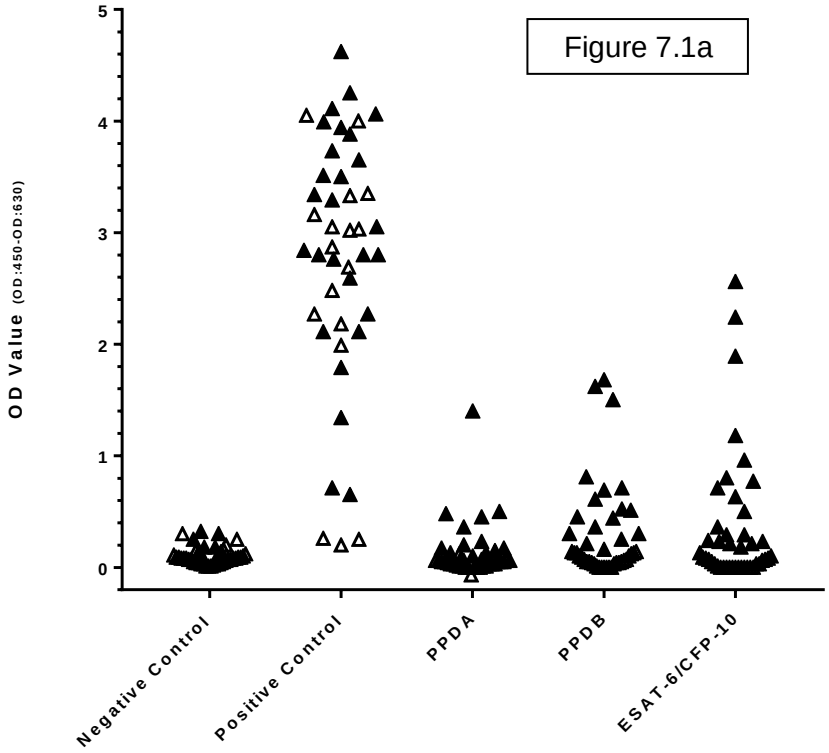
7.3.1 IGRA and TNF-α PBMC stimulation assays

In total, 164 dogs at high risk of subclinical *M. bovis* infection were tested by PBMC IGRA, results are shown in Figure 7.1a. Only 11 tests failed and required repetition to provide a viable result; the most frequent reason for this (n=6) was that there was a statistically insignificant response to the PMA/Ca positive control; in some cases there was clotting of the blood sample which precluded the isolation of PBMC (n=4), and one test required repetition due to significant disagreement between replicate ELISA values.

Of the 164 high risk dogs, 85 (52.0%) were found to be test positive by the case definition (positive to any of PPDA, PPDB or ESAT-6/CFP-10). Seventy seven (90.1%) of these dogs displayed a significant response bias to PPDB over the PPDA response*, i.e.* 47% of all HRG dogs tested displayed a response pattern indicative of MTBC infection. Within this group of 77 dogs, 48 (62.3%) also responded to the ESAT-6/CFP-10 combination*, i.e.* a response pattern

highly suggestive of *M. bovis*. In addition, five test positive individuals responded to ESAT-6 and CFP-10 and no other test antigens whilst another three responded only to PPDA.

The IGRA results for the 74 LRG and TB-free dogs are shown in Figure 7.1b. Among these dogs 14 (19.4%) showed a significant antigen-specific response according to the case definition (positive to any of PPDA, PPDB or ESAT-6/CFP-10). However, in contrast to the HRG, 12 (85.7% of these 14) of these responses were biased to PPDA compared to PPDB, suggestive of exposure to/infection with environmental mycobacteria rather than MTBC species. Only two animals in this group showed a positive bias to PPDB above PPDA, indicative of MTBC infection, suggesting a test specificity might be 97.3%. Neither of these dogs responded to the ESAT-6/CFP-10 peptides. Retrospective investigation identified that these two dogs had been previously cohabitant with the HRG. Were these dogs to be removed as dangerous contacts the test specificity would be higher at ~100%.



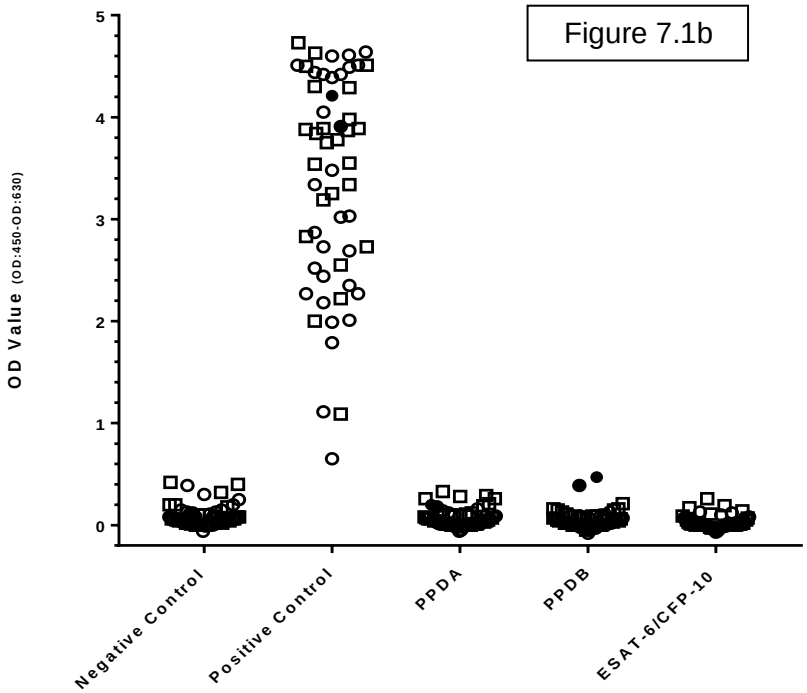


Figure 7.1: Results of the PBMC IGRA test for 164 HRG (Δ) (Fig. 7.1a), and 32 dogs from the LRG (○) and 42 from the TB-free group (□) (Fig. 7.1b) dogs. Each symbol shows the mean OD value of duplicate IFN-γ ELISA absorbance measurements for an individual dog. Solid symbols show a positive test where PPDB>PPDA within the low risk and TB-free groups.

The PBMC culture supernatants from 45 of the HRG dogs were assessed for antigen-specific TNF-α (Figure 7.2). Three of the 45 dogs had statistically significant quantities of TNF-α in the supernatant of cells stimulated with PPDB compared to the negative control condition. The same three animals, along with an additional 25 individuals, were found to have significant levels of TNF-α in the supernatant of PBMC stimulated with the ESAT-6/CFP-10 peptide cocktail.

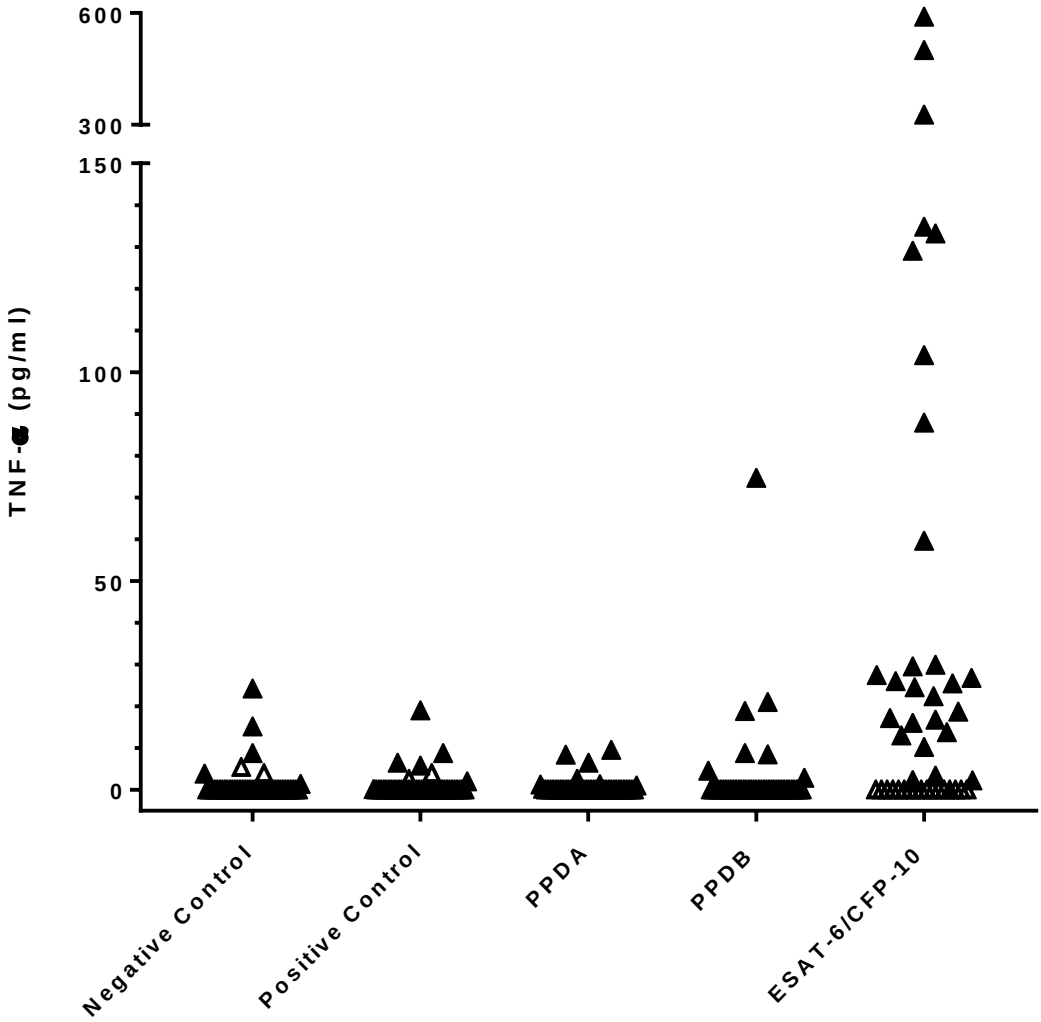


Figure 7.2: Results of TNF-α ELISA conducted on the same PBMC supernatant as the IGRA tests in a randomly assigned sub-group of 45 high risk group dogs. Each symbol shows the mean OD value of duplicate TNF-α ELISA absorbance measurements for an individual dog. Solid symbols show a positive test to ESAT-6/CFP-10 or where PPDB>PPDA.

Comparing the concentration of antigen-specific TNF-α produced in response to ESAT-6/CFP-10 stimulation only with the overall IGRA results (positive with either a PPDB bias or ESAT-6/CFP-10 response), showed that these two tests, interpreted in parallel, identified 29 (64.4%) test positive and 16 test negative individuals. These tests showed very strong agreement (Cohen's kappa coefficient, κ, =0.83).

However, the mitogen PMA/Ca used as a sample positive control for the IGRA test did not stimulate the production/secretion of canine TNF-α. As the HRG dog stimulations could not be re-run to include an additional mitogen, we compared the IFN-γ and TNF-α production from three healthy dog PBMC samples stimulated with a cocktail of PMA/Ca and ConA. In this instance TNF-α and IFN-γ were both produced (Figure 7.3). Therefore the lack of TNF-α response to the PMA/Ca positive control in the HRG group does not invalidate the positive antigen-specific responses observed to PPDB and ESAT-6/CFP10, rather it indicates that the positive control was not optimal and that future assays to measure this, and potentially other cytokines should consider a wider mitogen cocktail as a sample positive control.

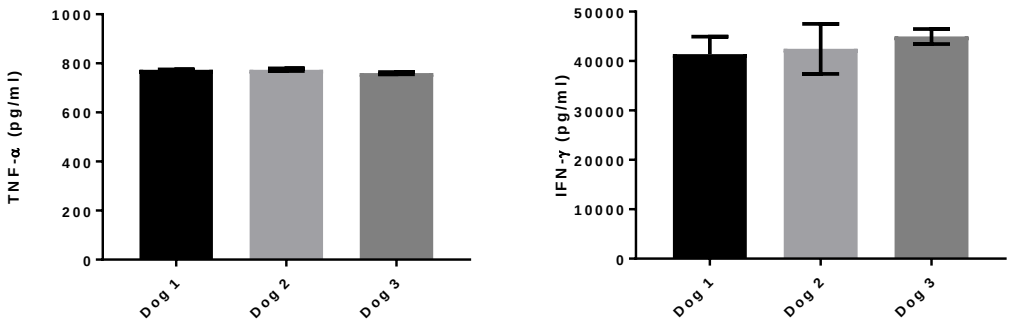


Figure 7.3: Concentration (replicate mean; error bars show standard deviation) of TNF-α (left) and IFN-γ (right) produced by PBMC stimulated for four days at 37ºC/5% CO_2 with PMA/Ca/ConA. Addition of Con A to the mitogen mix induced TNF-α in all three dogs, whereas all previous PBMC incubations with PMA/Ca did not induce detectable TNF-α.

The same subset of 45 HRG dogs was tested using a whole blood IGRA (Figure 7.4). Overall responses were low, with three individuals showing responses indicative of MTBC infection (PPDB>PPDA), two of which also showed responses to peptide cocktail and ESAT-6 protein. Comparing these results with those from the PBMC IGRA showed poor agreement between the two tests (κ= 0.2).

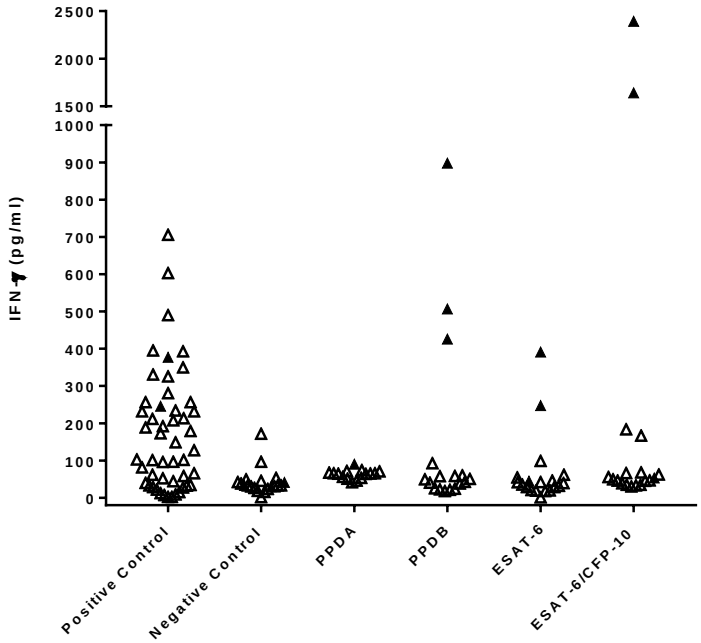


Figure 7.4: Results of the whole blood IGRA test for a randomly assigned sub-group of 45 of the high risk group of 164 dogs. Concentrations are calculated using the mean of duplicate IFN-γ ELISA absorbance measurements against the standard curve. Solid symbols show a positive test to ESAT-6/CFP-10 peptides or ESAT-6 or where PPDB>PPDA.

7.3.2 Serological Assays

The Idexx *M. bovis* Ab ELISA (Figure 7.5) showed a statistically significant difference between the OD values for the HRG (n=163) and TB-free (n=45) dog groups (Mann Whitney U = 880.5, P<0.001). In deciding upon a preliminary test cut-off for the canine samples tested with the Idexx *M. bovis* Ab assay, we took into account both the cattle Idexx test protocol and the modified Idexx test as applied to camelid TB testing at APHA, both of which approximate to an OD cut-off value of 0.3 to provide high test specificity. Application of a cut off value of 0.3 to the canine samples tested with the Idexx *M. bovis* Ab test, the data resulted in 100% test specificity for this TB-free group, and identified 12 test-positive dogs within the HRG (7.4%).

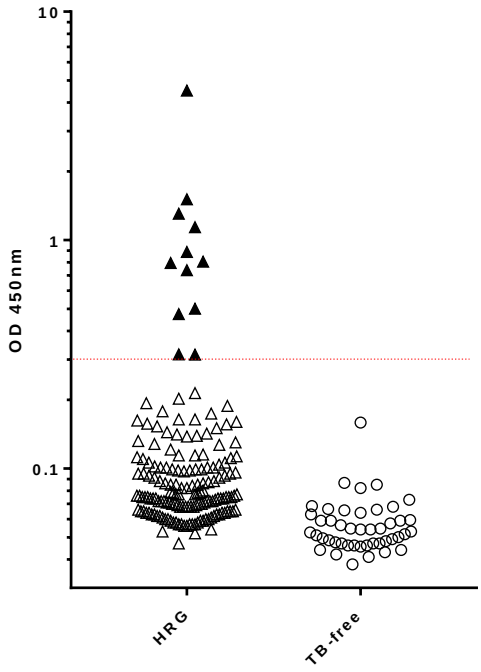


Figure 7.5: Serology results for dogs in the high risk group (HRG, n=163) and TB-free group (n=45) obtained using the Idexx *M. bovis* Ab test. Results shown are the mean OD values of duplicate measurements for individual dog samples at 450nm absorbance. The test-positive cut off value of OD 0.3 is shown by a horizontal line. Solid symbols show test-positive samples.

The DPP VetTB test (Figure 7.6) showed significantly higher RLU results for the HRG compared to the TB-free dogs (Mann Whitney U = 2731, P=0.003). In deciding upon preliminary cut-offs for the MPB83 and ESAT6/CFP-10 antigens in this test, a preliminary ROC analysis for the MPB83 RLU values suggested a cut-off of >95 to provide a specificity of 100%. A similar analysis for ESAT-6/CFP-10 was not viable due to the very low number (n=2) of sero-positives; however, there was a clear divide between these two sero-positives and test-negatives, of between 40-80 RLU, therefore >60 RLU was chosen as the cut-off for the ESAT-/CFP-10 antigen line for the purpose of this study. Applying these cut-offs to the study cohorts provided a 100% specificity, and identified 11 HRG dogs (6.7%) as test-positive; nine of these were positive to MPB83 only, whilst two were positive to ESAT-6/CFP-10 only.

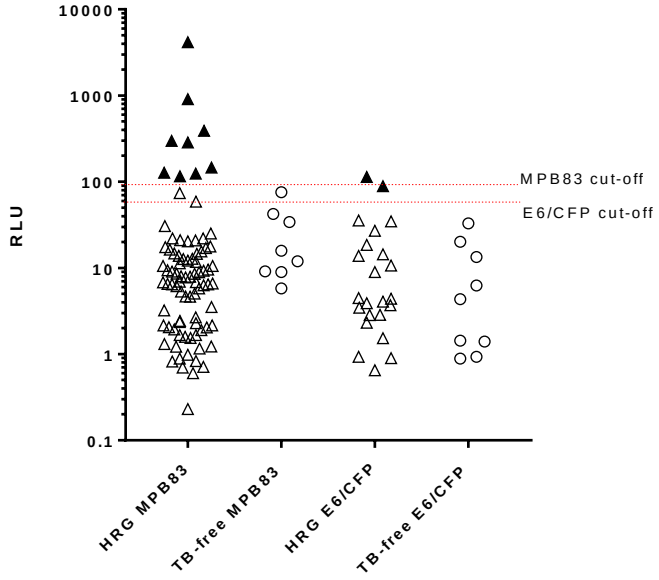


Figure 7.6: Serology results for dogs in the high risk group (HRG, n=163) and TB-free group (n=45) obtained using the DPP VetTB Assay. Results given as Optricon relative light units (RLU). The test-positive cut off values for each antigen are shown by the horizontal lines at 95 RLU for MPB83 and 60 RLU for ESAT-6/CFP-10 (E6/CFP). Solid symbols indicate a positive sample. As the axis is logarithmic, only values greater than zero can be plotted. For this graph, 291 values overall were zero or negative and are therefore not included on the graph.

The comparative ELISA (Figure 7.7) found generally higher responses to PPDA than to PPDB in the TB-free population. The test cut-off was calculated as the mean OD [PPDB-PPDA] of the TB-free group plus three standard deviations, providing a cut-off value of 0.14. Using this threshold gave a 100% specificity and identified 27 test-positive HRG dogs (16.6%).

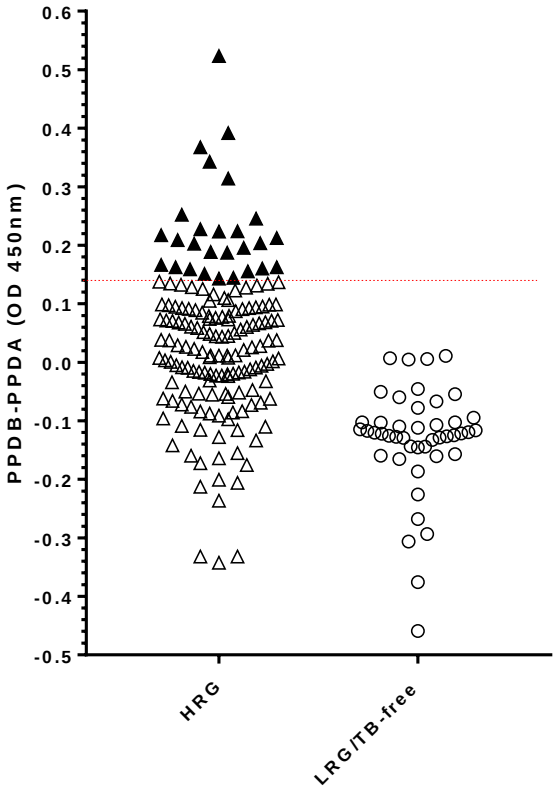


Figure 7.7: Serology results for dogs in both the high risk group (HRG, n=163) and TB-free group (n=45) obtained using a comparative PPD antibody ELISA. Results shown are mean values of duplicate measurements calculated by subtracting the PPDA $OD_{480-630}$ from the PPDB $OD_{480-630}$ values. The test-positive cut off (OD 0.14) is shown by a horizontal line. Each symbol represents one dog sample. Test-positives are shown as solid symbols.

7.3.3 Serum IFN-γ & TNF-α ELISA

No statistical difference was found between the quantities of either TNF-α or IFN-γ present in the peripheral circulation of the dogs comprising the HRG and LRG (data not shown).

7.3.4 Haematology and Serum Biochemistry

Standard haematological and serum biochemical analysis was conducted on samples collected from half (n=83) of the HRG dogs, selected at random. The only haematological abnormality identified frequently was an increase in the total leukocyte count (n=22, 26.5%); in most cases this was due to the presence of a mature neutrophilia (n= 18, 81.2%) while band

neutrophils were also present in a minority of cases (n=4, 4.8%). One dog was found to have each of eosinophilia, monocytosis and reticulocytosis.

Only five dogs of the 83 tested (6.0%) were found to have all serum biochemical tests within the reference interval (RI). The most frequently detected abnormality was elevated serum urea which was above RI in 44 of the dogs (53.0%), three of which also had elevated bile acid concentrations. Total calcium was low in 18 samples (21.7%) whilst alkaline phosphatase (ALP) activity was reduced in 25 samples (30.1%) and increased in six. More than a quarter (22, 26.5%) of dogs were found to have elevated globulin levels; 20 of them were elevated enough to increase the total protein concentration of the serum to greater than RI with normal albumin concentrations. No association was found between these changes and the results of the other tests.

7.4 Discussion

Almost every species of mammal, including the domestic dog, is susceptible to infection with MTBC organisms, including *M. bovis* (Brosch, 2002; Drewe, 2010; Miller, 2012; Parsons, 2013; Angkawanish, 2013). Canine disease due to *M. bovis* has been documented in a number of clinical case reports over many decades, with several reports demonstrating inter-species transmission, including to and from humans (Snider, 1971; Liu, 1980; Gay, 2000; Ayele, 2004; Ellis, 2006; Van Der Bürgt, 2009; Shrikrishna, 2009; Posthaus, 2011; Pesciaroli, 2014; Park, 2016, Szaluś-Jordanow, 2016). Despite this, *ante-mortem* diagnostic testing for TB in the dog is limited. There is a single study describing the use of an IGRA for the detection of *M. tuberculosis* in a group of 40 dogs living in close contact with sputum-positive pulmonary TB owners (Parsons *et al.* 2012). To our knowledge, no other *ante mortem* tests have been evaluated as effective for the diagnosis of canine MTBC infections; intradermal TST for both *M. bovis* and *M. tuberculosis* is unreliable, as are complement fixation and haemagglutination methods (Snider *et al.* 1971; Snider *et al.* 1975; Parsons *et al.* 2012 Broughan *et al.* 2013b).

In the UK, *M. bovis* infection is endemic, with the highest prevalence in the counties comprising the HRA, and lower but still significant prevalence within the Edge Area, defined by the

incidence of bovine infections. In these areas many species other than cattle are also infected with *M. bovis* and it was from within the Edge Area that we previously reported an outbreak of *M. bovis* disease in a pack of kennelled foxhounds (Delahay *et al.* 2002, O'Halloran *et al* 2018). The lack of reliable diagnostic assays meant that the prevalence of subclinical *M. bovis* infection within the group of exposed animals was challenging to determine, and potentially led to the outbreak lasting longer, and costing more to bring under control, than if such a test or tests had been available.

To begin to address this shortcoming, we have in this study applied commercially available tests produced for other species (Idexx for cattle, DPP VetTB for cervids) with an in-house serology test, and cellular cytokine release assays (IFN-γ and TNF-α) using commercial reagents, for their ability to detect subclinical infections in dogs from both high and low expected prevalence populations. All of the tests chosen evaluate the host immune response rather than relying on direct pathogen identification or isolation. Previous studies have demonstrated that the major immunological consequences of MTBC infections are broadly conserved across host species (Cousins and Florisson, 2005; Broughan, *et al.* 2013b). Once mycobacteria are phagocytosed, macrophages and dendritic cells present antigen from lysed bacilli to $CD4^+$ and $CD8^+$ αβ as well as γδ T-cell subsets in the context of cell surface MHC class II molecules. The reciprocal activation between cells of these immunophenotypes, through the secretion of IFN-γ and TNF-α along with other cytokines, generates the classical cell mediated response required for the control of an intracellular pathogen.

The first diagnostic test we assessed was an adapted IGRA, identifying animals with IFN-γ producing antigen-specific T-cells. Similar to domestic cats, lion, alpaca and other new world camelids, we found that it was necessary to isolate PBMC prior to antigen stimulation in order to reliably generate interpretable test data under these conditions (Hesketh *et al.* 1994; Gutierrez *et al.* 1998; Cousins and Florisson, 2005; Rhodes *et al.* 2008a*;* Leem *et al.* 2018). This was due to the failure of cells within the whole blood of these species to respond significantly to either positive control or antigen stimulation, unlike the whole blood responses

observed in cattle, goats and humans (Vordermeier *et al.* 2006; Vordermeier *et al.* 2007; Bezos *et al.* 2018; Leem *et al.* 2018).

Examining the responses generated by the HRG dogs, we found that 77 (47.0%) showed a PPDB biased response indicating subclinical MTBC infection; compared to the LRG groups where only two animals (2/32, 6.3%) showed this response; interestingly, these were the two dogs most in-contact with the HRG hounds whilst no animals in the TB-free group showed an MTBC response. These results suggest this test method and interpretation has a high specificity of 97.3-100%, which is within the range of 90-100% consistently reported for IGRA assays in cattle, cats and goats (Vordermeier *et al.* 2006; Rhodes *et al.* 2012; Bezos *et al.* 2018). Of the 77 HRG dogs showing a PPDB biased response, 62% simultaneously showed a significant response the ESAT-6/CFP-10, strongly suggestive of infection with an RD-1 positive MTBC organism; this is slightly lower than the proportion reported for both cattle (~85% in the high specificity IGRA where both PPD and peptide must be positive, and 82% for peptide alone as a diagnostic, Vordermeier *et al.*, 2016) and cats (80% but only ten cats were tested, Rhodes *et al.,* 2011). This difference may reflect a lesser immunological significance of these antigens in canine infections and/or may be an artefact of the unknown incubation time from infection to blood sampling in the HRG dogs; had infections been allowed to continue to incubate, the number of dogs responding to these peptides may have increased.

A further five dogs in the HRG only responded to the peptide cocktail ESAT-6/CFP-10. With a lack of an antigen-specific PPDB response, it is questionable whether these animals are truly infected with *M. bovis* or not, since some NTM (notably *M. kansasii* and *M. leprae,* among others), encode orthologs of these proteins, and as the HRG in this study comprised working Foxhounds there was significant potential for them to be exposed to/infected with confounding NTM. However, infected cattle are known to respond to ESAT6/CFP-10 in the absence of a positive PPD response, and in a setting of confirmed *M. bovis* infection such as the HRG dogs, a positive response to these peptides suggests an individual at high risk of being infected. Furthermore, no animals in the LRG/TB-free groups demonstrated ESAT-6/CFP-10 responses, despite many having similar opportunity for NTM sensitisation as the HRG. So,

whilst the potential for confounding influence of NTM is important for interpreting individual animal responses, at a group level the data presented here would suggest that a response to ESAT-6/CFP-10, even alone, is sufficient to indicate high risk of *M. bovis* challenge.

In our previous assessment of test responses, individual dogs with a significant response to any test antigen were considered test-positive (O'Halloran *et al.* 2018); this included three dogs that responded to PPDA alone. Analysis of the LRG tests found that this response occurred in 12 of the 74 dogs (16.2%) suggesting that this should not be seen as indicative of being at risk of *M. bovis* infection. Therefore, the definition of IGRA-positive animals in the context of subclinical *M. bovis* infections, should be revised to consider only individuals that show a statistically significant response above the media control to PPDB that is greater than the PPDA response, and/or those who respond significantly to the ESAT-6/CFP-10 combination.

The diagnostic sensitivity of assessing antigen-specific TNF-α responses has only recently been tested clinically and on relatively small populations of *M. tuberculosis*-infected people, with inconclusive results; however, it shows promise for discriminating between active and latent disease (Harari *et al.* 2011; Wang *et al.* 2013). The approach we used, combining two ELISA cytokine measurements (IFN-γ and TNF-α) on the same stimulated cell supernatants, identified additional test-positive individuals suggesting that a dual cytokine test approach may have a higher diagnostic sensitivity without the need to take any additional blood from the animals being tested. However, going forward there would need to be a modification of the positive mitogen control to include ConA, since PMA/Ca alone did not stimulate canine TNF-α release.

The detection of specific antibody responses has been useful in the development of new diagnostic tests for mycobacterial infections, particularly for species where repeat capture of the same individual at a fixed time point is difficult, and therefore precludes the use of TST (Gao *et al.* 2007; Lesellier *et al.* 2008; Bezos *et al.* 2018) or where reagents for IGRA tests are

not available. We therefore examined the antibody responses of dogs using a combination of serological tests already in use for other species.

As expected, the total number of test-positive animals identified by the serological assays was significantly lower than for the PBMC IGRA; 16.5%, 7.4% and 6.7% for PPD ELISA, Idexx ELISA and DPP VetTB tests respectively, compared to 52% IGRA-positive in the HRG dogs).

However the simplicity of the antibody tests compared with the IGRA test does provide relative speed and a lower test cost compared to the IGRA, which could facilitate an initial blood test screening of large numbers of animals in confirmed breakdown situations, and/or provide a serological option where blood sampling and rapid, temperature controlled transport (for IGRA) may not be possible.

Of the three antibody tests the comparative PPD ELISA found a much higher number of test-positive dogs, 27 compared to 12 (Idexx) and 11 (DPP VetTB). Whether this is due to PPD providing the largest epitope repertoire of all assays tested, or whether the test cut-off is not optimal remains to be explored. Many LRG/TB-free dogs had a PPDA-biased response resulting in a very low test-cut-off (0.14) compared to the Idexx ELISA (cut-off 0.3). However, 25 of the 27 comparative PPD positive dogs were positive to at least one other test (PMBC IGRA [20], DPP VetTB Assay [7] and/or Idexx ELISA [6]) and so this does provide confidence in the PPD ELISA results.

Evaluating all of the test responses for each individual dog, it became clear that a small minority of individuals showed much higher responses to the tests than the rest of the test positive population on each assay. Further examination showed that this was consistently due to three individuals for the cell based and cytokine assays, and a single individual for the antibody based assays. The relevance of the magnitude of this test response is unclear. There is conflicting evidence in the scientific literature as to whether the concentration of cytokine in serum and/or produced in *in vitro* diagnostic assays is proportionate to the severity or progression of clinical disease *in vivo* (Hasan *et al.* 2009; Ruhwald *et al.* 2009; Jonnalagadda *et al.* 2013; Xu *et al.* 2018), whilst there is some evidence indicating that ESAT-6/CFP-10-

specific IFN-γ responses correlate with disease severity in cattle (Vordermeier *et al.* 2002) and that the titre of detectable antibody correlates with the occurrence of a lesioned phenotype (Roos *et al.* 2016). This may be true for dogs; however, data in this study did not support this view, possibly because of to the subclinical nature of the HRG tested.

Haematological abnormalities were identified infrequently in the 83 dogs in the HRG that were tested. The high total leukocyte counts, mature and band neutrophilia seen in a small number of dogs did not correlate with any of the mycobacteria-specific diagnostic tests; they probably reflect an unknown inflammatory stimulus in these dogs.

Serum biochemistry of the same dogs revealed multiple frequent abnormalities. The most frequent, elevated urea concentration, likely reflects the high protein diet of the working hounds as they were fed raw meat, rather than any underlying pathology. Half of the 22 dogs with hyperglobulinaemia were also found to be positive to at least one of the serology assays indicating that in some cases hyperglobulinaemia may have been related to the presence of antigen-specific antibodies. However, the overall specificity of hyperglobulinaemia with respect to the remainder of the tests was poor.

This study was unable to evaluate test sensitivity as too few confirmed infections were identified – in total seven confirmed clinical cases of *M. bovis* infection occurred during the course of the kennel outbreak. Due to the severity of the disease, these affected animals were euthanased on welfare grounds before diagnostic blood samples could be taken. The diagnosis of *M. bovis* infection in these animals was confirmed by mycobacterial culture of visibly lesioned tissues collected *post-mortem* in each of these cases. The rate of false negative test results (type II error) in this study is therefore also unknown.

In summary, this study demonstrates, for the first time, the utility of two cellular (IGRA and TNF-α) and three serological assays (DPP VetTB, Idexx and comparative PPD ELISA) for detecting sub-clinical *M. bovis* infections of dogs. Whilst the data suggest high test specificity for all assays evaluated, further work is needed to robustly validate the specificity of the tests, and investigate test sensitivity using sufficient numbers of dogs of confirmed infection status.

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Chapter 8: Bayesian Latent Class Estimation of the Performance Parameters of Diagnostic Tests to Identify *Mycobacterium bovis* Infected Dogs.

Abstract

There are currently no diagnostic tests available for the *ante-mortem* identification of *Mycobacterium* (*M.) bovis* infections in domestic dogs (*Canis lupus familiaris*). We previously reported the use of a combination of eight tests to identify infected individuals during an outbreak of *M. bovis* tuberculosis in a pack of Foxhounds (Chapters 5 and 7); four of these tests were shown to be promising for the detection of infected animals. A retrospective Bayesian latent class analysis of the combined four test results of 164 hounds was conducted in order to estimate the sensitivity, specificity, positive and negative predictive values of each test as well as the prevalence of infection in the pack. The cell-based interferon gamma release assay (IGRA) was shown to be the most sensitive test and had the highest positive and negative predictive values with moderate specificity. The three serological assays were shown to have higher specificities but lower sensitivities than the IGRA. Of the serological tests, the comparative purified protein derivative (PPD) ELISA performed best for all parameters estimated, followed by the Idexx *M. bovis* Ab ELISA with the DPP VetTB assay performing least well. This differential performance between the cell-based IGRA and serological assays suggests that testing protocols combining both approaches may be most useful for identifying and controlling future outbreaks.The model was improved by the addition of informed priors but was not significantly affected by the inclusion of covariance between the serological assays. This study provides useful test performance data that could not have been derived using a traditional frequentist approach. Further work is now needed to improve the credibility of the initial estimates produced in this analysis by expanding the population of dogs with test data available, particularly those whose infection status is definitively known.

8.1 Introduction

Tuberculosis due to infection with *Mycobacterium (M.) bovis* is a chronic zoonotic infectious disease currently endemic among cattle and wildlife populations in England and Wales[1-3]. The major impact of infection is within the production cattle population and disease control measures cost the UK government in excess of £100 million annually[3,4]. Despite the implementation of a strict statutory testing policy, disease elimination has proven difficult; likely due to the complex and multifactorial nature of the host, pathogen and environmental interaction which results in disease transmission before infected animals can be detected[3-5]. Further to this, there are a number of significant maintenance and spill over hosts including the Eurasian badger (*Meles meles*)[4], various deer species[6,7], North American camelids[8,9] as well as companion animals including both the domestic cat (*Felis catus*)[10] and dog (*Canis lupus familiaris*)[11].

Recently, we investigated a large outbreak of *M. bovis* tuberculosis in a large working pack of Foxhounds in England[12]. At the time there was discussion in the veterinary and national press regarding whether working hounds contribute to the contamination with *M. bovis* of the environment where cattle and other susceptible species may be co-habitant or use as grazing pasture[13-17]. A complete and robust epidemiological investigation conducted jointly by our group and the Animal and Plant Health Agency (APHA) concluded that there was no evidence to suggest that dogs play a significant role in the persistence of *M. bovis* in England and Wales or that scent and drag hunting with dogs contributes to the spread of bovine tuberculosis amongst cattle or wildlife (reported in this thesis, Chapter 5)[12].

Whilst the risk posed by hounds was being evaluated, there was a concurrent time delay in establishing control over the outbreak as there are no validated *ante-mortem* diagnostic tests for canine *M. bovis* infections[12]. The only diagnostic test recognised in statute, and therefore the test designated as the 'gold standard', is *post-mortem* specialist mycobacterial culture of visibly lesioned (granulomatous) tissues[18]. However, the concept of a "gold standard" in this case can be considered misleading; the term was only intended to be applied to a test which

has (near) perfect performance relating to both sensitivity (Se) and specificity (Sp)[19]. Logically, the isolation of viable *M. bovis* organisms from lesions obtained from cadavers gives culture an assumed (near) perfect Sp for infection with that organism, provided rigorous laboratory protocols are in place to prevent sample contamination during handling. The Se of culture for canine derived samples has not been assessed, but in domestic cats the overall Se of all mycobacterial culture is under 50%[20] whilst the Se of *M. bovis* culture is reported to be approximately 80% in cattle[21]. These data suggest that it may be inappropriate to refer to *post-mortem* tissue culture as the 'gold standard' test in the outbreak of *M. bovis* studied herein. Furthermore, during the investigation, it was only practically possible to conduct *post-mortem* examinations on a subset of the hounds that were euthanased, meaning that in most cases 'gold standard' test data were not available. In addition, in a number of samples no growth of *M. bovis* was observed from tissue samples after 16 weeks[12] which is difficult to interpret for a test with unknown Se.

Taking this into account made accurate estimation of the performance parameters for the testing strategy that we used challenging whilst relying on frequentist analytical methods to compare our results to mycobacterial culture. An alternative method for such an analysis in the absence of an appropriate or accurate gold standard test is to use a Bayesian latent class modelling approach[22-24]. This statistical methodology does not require the true infection status of an individual to be known but instead models this as a latent (unknown) variable along with the test performance parameters[22-24]. Methods adhering to a Bayesian rather than frequentist framework have been successfully utilised since the development of the two-test, two-population model introduced by Hui and Walter in 1980[24]. Since then, the World Organisation for Animal Health (the OIE) has encouraged the use of these models; particularly in cases, as presented here, where the acquisition of sufficient numbers of samples from animals of known infection-status would be extremely difficult due to the low prevalence of disease, where current gold standard diagnostic testing methods have known limitations and/or the experimental infection of animals to produce robust test performance data would be considered unethical[25].

The methodology established by Hiu and Walter is dependent on the critical assumptions that; a) when comparing multiple populations, the underlying disease prevalence differs between them, but that b) the test performance is the same across them and, c) the (two) tests are conditionally independent from one another[24]. In our outbreak scenario, however, there was only a single epidemiological population without a second that we could be sure adequately satisfies these assumptions. This limitation is not uncommonly encountered when studying infectious diseases as it is inherently challenging to sample populations of animals which have differing infection prevalence but do not respond differently to the tests being conducted[26]. Therefore, an extension to the original methodology has been proposed whereby three or more tests are performed on a single study population[26,27], an approach which has previously been adopted for the estimation of test performance relating to bovine *M. bovis* infections[5].

The aim of the study presented here was to analyse a panel of four tests that we have previously established as promising in their application to detect subclinical *M. bovis* infections in dogs using samples collected *ante* mortem[28] (reported in Chapter 7) and employ a Bayesian latent class analysis to estimate the performance parameters (Se, Sp, positive predictive value [PPV] and negative predictive value [NPV]) of each test and the underlying infection prevalence in the population.

We demonstrate that the cell-based interferon gamma release assay (IGRA) was shown to be the most sensitive test and had the highest positive and negative predictive values with moderate specificity. The three serological assays were shown to have higher specificities but lower sensitivities than the IGRA.

8.2 Materials and Methods:

8.2.1 Study Population & Test Data

We previously investigated a fulminant disease outbreak caused by *M. bovis* infection within a large group of kennel-housed working Foxhounds in England[12] (Chapter 5). A total of 164 hounds were sampled over the course of the outbreak and so had test data available for

analysis in this study[12]. The samples were previously analysed using eight test protocols that were either previously developed for the detection of *M. bovis* infections in non-canid species or newly developed by our group[12,28].

We demonstrated that four of these assays produced results which overall gave were able to distinguish between high and low risk groups of individuals, indicating that they had good potential to be developed into diagnostic assays[28]. These included a cell-based interferon gamma release assay (coded here as "IGRA"), the Idexx *M. bovis* Ab ELISA (Idexx, USA; coded as "Idexx"), the DPP lateral flow VetTB Assay for Cervids (Chembio, USA; coded as "DPP") and a newly developed comparative purified protein derivative (PPD) ELISA (coded as "CompPPD")[28].

8.2.2 Bayesian Latent Class Analysis

As previously described, we tested 119 *M. bovis*-free and *M. bovis*-'low risk' dogs using the same protocols as the high risk dogs in order to determine specific cut-off values for each test methodology[28]. We therefore ascribed each of the 164 hounds a binary positive or negative status for each of the four tests allocating each individual to one of 16 possible combinations of positive and negative test results.

Bayesian latent class models were used to estimate diagnostic test Se, Sp, PPV, NPV and the prevalence of infection. The computations were implemented using WinBugs version 1.4.3.

All models utilised a four-test one-population approach. As no data are available on the performance of these assays in domestic dogs, model one was coded (Appendix 2) to include uninformative flat uniform priors; beta (1, 1). Throughout the study all models were run for 100,000 iterations with the first 5000 discarded as a "burn in". Chain convergence after the initial burn-in was assessed by visual inspection of the Markov chain Monte Carlo outputs.

For tests to be of potential benefit to clinicians, the PPV and/or NPV of a test needs to at least be greater than 50% (*i.e.* better than chance) or else no decisions can be made based on the test result. Subsequently to evaluating model one, informed priors were included into a second

model in order to improve the clinical utility of the posterior estimates. The values assigned were based on data obtained from a review of relevant bovine literature[29-35]. The sensitivity of IGRAs reported in the literature ranged from 55% to 93% with 74% most frequently reported and their specificity ranged from 89% to 100% with a consensus around 95%[29-35]. These values were converted into beta (15.04, 5.28) and beta (64, 3.7) values respectively; the beta distributions and designated initial values used for all parameters in model two are provided in Table 8.1. Where published data were unavailable, such as for the in-house comparative ELISA, the posterior estimates obtained from model one were used as *a priori* information for models two and three.

Table 8.1: The beta distributions and designated initial values for each variable used in models two and three.

Variable	Beta Distribution	Initial value
SeIGRA	(15.04, 5.28)	Se=0.74
SpIGRA	(64, 3.7)	Sp=0.95
SeDPP	(1, 1)	Se=0.65
SpDPP	(1, 1)	Sp=0.98
SeCompPPD	(1, 1)	Se=0.10
SpCompPPD	(1, 1)	Sp=0.20
SeIdexx	(98.77, 261.40)	Se=0.5
SpIdexx	(137.64, 6.81)	Sp=0.97

In models one and two, we had followed the implicit model assumption that the different tests are conditionally independent of one another. However, as three of the four tests are serological assays, albeit of different types, we included undefined covariance terms into model three to allow for possible interdependence between these test results to be accounted for. Model three included the same prior distributions as model two which are provided in Table 8.1.

8.3 Results

Between December 2016 and August 2017 we tested a total of 164 Foxhounds kept at a single kennel premises to try and identify all of the individual dogs infected with *M. bovis* during a tuberculosis outbreak[12]. The combination and frequency of the results generated by the four testing protocols retrospectively analysed in this study are shown in Table 8.2.

Table 8.2: Test result categories for the 164 dogs included in this study in descending order of frequency.

IGRA	Idexx Ab ELISA	DPP VetTB assay	Comparative PPD ELISA	Frequency (Total = 164)
Negative	Negative	Negative	Negative	66
Positive	Negative	Negative	Negative	62
Positive	Positive	Negative	Negative	15
Positive	Positive	Positive	Positive	4
Negative	Positive	Positive	Negative	3
Negative	Negative	Negative	Positive	3
Positive	Positive	Negative	Negative	2
Negative	Negative	Positive	Negative	2
Positive	Positive	Positive	Negative	2
Positive	Negative	Positive	Negative	1
Positive	Negative	Negative	Positive	1
Negative	Negative	Positive	Positive	1
Negative	Positive	Positive	Positive	1
Positive	Positive	Negative	Positive	1
Negative	Positive	Negative	Positive	0
Positive	Negative	Positive	Positive	0

The posterior parameter estimates for all three models generated in this study are provided in Table 8.3. In model one, the IGRA test had the greatest Se with a median posterior estimate of 51% (95% credibility interval [CrI] 42-59%). The IGRA and the comparative PPD ELISA were the only assays shown to have a clinically useful PPV, defined as greater than 50%, with a median estimate of 89% (CrI 69-96%) and 55% (CrI 5-79%); however, only the IGRA had the full 95% credibility interval above 50%. The Idexx *M. bovis* Ab ELISA was shown to have a specificity estimate of 56%, however the PPV (40%) and NPV (6%) estimates were too low for this to be considered a clinically useful test. All of the remaining test parameter estimates

in model one had posterior parameter estimates below 50% and broad credibility intervals which further indicated the need to include *a priori* information into the model.

Table 8.3: Interferon gamma release assay ('IGRA'), Idexx *M.bovis* Ab ELISA ('Idexx'), DPP VetTB assay ('DPP') and Comparative PPD ELISA ('CompPPD') diagnostic test parameter estimation based on the test results of 164 individuals. Posterior parameter estimates are given as proportions. 95%CrI: 95% credibility interval, Se: test sensitivity, Sp: test specificity, PPV: test positive predictive value, NPV: test negative predictive value.

Variable	**Model one**			**Model two**			**Model three**		
	Mean	Median	95% CrI	Mean	Median	95% CrI	Mean	Median	95% CrI
SeIGRA	0.51	0.51	0.42-0.59	0.68	0.64	0.51-0.90	0.72	0.77	0.53-0.92
SpIGRA	0.38	0.37	0.13-0.67	0.57	0.55	0.43-0.79	0.59	0.59	0.44-0.80
PPV IGRA	0.89	0.88	0.69-0.96	0.57	0.74	0.39-0.98	0.58	0.75	0.39-0.98
NPV IGRA	0.08	0.08	0.02-0.18	0.56	0.82	0.08-0.99	0.56	0.82	0.08-0.99
SeDPP	0.03	0.02	0.00-0.07	0.31	0.15	0.02-0.83	0.24	0.18	0.16-0.53
SpDPP	0.31	0.29	0.02-0.70	0.56	0.86	0.01-0.99	0.62	0.76	0.48-0.73
PPV DPP	0.25	0.23	0.02-0.58	0.57	0.56	0.17-0.96	0.63	0.57	0.11-0.99
NPV DPP	0.04	0.04	0.03-0.16	0.51	0.59	0.04-0.98	0.51	0.59	0.04-0.98
SeCompPPD ELISA	0.10	0.12	0.01-0.17	0.38	0.36	0.10-0.77	0.42	0.32	0.12-0.74
SpCompPPD ELISA	0.20	0.19	0.18-0.49	0.55	0.76	0.02-0.97	0.56	0.77	0.02-0.97
PPV CompPPD ELISA	0.51	0.55	0.05-0.79	0.59	0.63	0.19-0.89	0.59	0.61	0.19-0.89
NPV CompPPD ELISA	0.03	0.02	0.00-0.09	0.52	0.67	0.04-0.98	0.50	0.65	0.04-0.98
SeIdexx	0.03	0.03	0.00-0.07	0.27	0.14	0.03-0.74	0.24	0.17	0.03-0.74
SpIdexx	0.55	0.56	0.22-0.83	0.71	0.89	0.17-0.99	0.71	0.89	0.17-0.99
PPV Idexx	0.40	0.40	0.11-0.72	0.64	0.64	0.35-0.92	0.64	0.64	0.35-0.92
NPV Idexx	0.07	0.06	0.01-0.18	0.50	0.61	0.04-0.98	0.50	0.61	0.04-0.98
Prevalence of infection	0.89	0.90	0.78-0.96	0.53	0.55	0.05-0.96	0.53	0.55	0.05-0.96

Model two and three both included informed priors with respect to test performance. The parameter estimates generated from model two indicate, in agreement with model one, that the IGRA is the most sensitive of the tests analysed (median estimate 64%, CrI 51-90%) followed by the comparative PPD ELISA (median estimate 36%, CrI 10-77%), with the DPP lateral flow assay and the Idexx *M. bovis* Ab ELISA performing very similarly for this parameter estimate (DPP lateral flow median sensitivity estimate 15%, CrI 2-83%; Idexx *M. bovis* Ab ELISA median sensitivity estimate 14%, CrI 3-74%). By contrast, all three of the antibody detection assays were estimated to have greater specificities than the IGRA, which was estimated by this model to have a specificity of 55% (CrI 43-79%), ranging from 76-89% but with wide 95% credibility intervals such as 1-99% for the DPP lateral flow assay.

In model two, all tests were shown to meet our definition of a clinically useful test. The model two analysis again showed that the IGRA was the best performing test with a PPV estimate of 74% (CrI 39-98%) and a NPV estimate of 82% (CrI 8-99%). By these estimated performance measures the comparative PPD ELISA (PPV 63%, CrI 19-89%; NPV 67%, CrI 4-98%) and the Idexx *M. bovis* Ab ELISA (PPV 64%, CrI 35-92%; NPV 61%, CrI 4-98%) performed similarly to one another with the DPP lateral flow assay appearing to be the least useful with the lowest predictive value estimates (PPV 56%, CrI 17-96%; NPV 59%, CrI 4-98%).

In model three, covariance terms were included to allow for the possibility of conditional dependence between the antibody-based test assays. Overall bivariate correlations were very low between these three assays (ρ=0.16, 0.17 and 0.26 respectively) and including the terms into the model did not significantly alter most of the posterior parameter estimates; only the sensitivity estimate of the IGRA increased to a median estimate of 77% (CrI 53-92%) while the remaining median estimate values remained within 5% of those generated in model two. Further comparison of the deviance information criterion (DIC) values between model two and three showed only a small change (less than three) indicating that the inclusion of the covariance terms did not significantly improve the fit of the model to the data.

8.4 Discussion

Bayesian latent class analysis provides an accepted alternative methodology for estimating diagnostic test performance when an affordable, reliable and/or non-invasive reference standard does not exist[23-27]. These methods are particularly useful for cases of infectious disease which have long subclinical latent periods, such as tuberculosis; this is because the latent class models do not require the true infection status of any of the animals in the test population to be known[22]. The OIE has continuously endorsed the use of latent class models for the assessment of novel diagnostic test performance in animal populations in recent years[25]. Using this technique we modelled the test performance of four previously unevaluated diagnostic tests for their *ante mortem* accuracy to diagnose subclinical *M. bovis* infections in domestic dogs.

All three of the latent class models that we developed during this study consistently showed that the cell based IGRA test had the greatest sensitivity for detecting subclinical infections. As an *ex vivo* T-cell stimulation assay, this IGRA test detects an antigen-specific cell mediated immune response to mycobacteria which is typically formed in response to intracellular pathogens including *M. bovis*[36,37]*.* The IGRA test assays are therefore considered to be both sensitive and relatively specific indicators of infection[36,37]*.* Model three produced a median posterior sensitivity estimate for the canine IGRA of 77% (CrI 53-92%) for the detection of subclinical *M. bovis* infections. This result is similar to those reported for the approved human test to detect both latent and active *M. tuberculosis* infections, the QuantiFERON^{TM}-TB Gold IGRA test[36,38,39]. Two recent studies independently evaluated the Se and Sp of the QuantiFERON^{TM}-TB Gold test comparing its performance to the gold standard of tuberculin skin testing and found it be 75% (95% confidence interval 53.1–88.8%) sensitive and 80% (95% confidence interval 64.1–90.0%) specific respectively[38,39]. In addition, a study evaluating the sensitivity of IGRA testing in cats for culture positive *M. bovis* infections, using almost exactly the same methodology as the test evaluated here, found the Se to be 80% compared to the gold standard of specialist mycobacterial culture[40]. It is not possible to compare these performance data to cattle sensitivity studies evaluating the BOVIGAM^{TM} assay as recent

bovine sensitivity data was used as prior information to build model three. Overall therefore, this study demonstrates that the canine IGRA here performs in line with assays designed to detect tuberculous infections in other animals and can therefore be considered a sensitive test.

This study also analysed the performance of three serological assays for their use in canine populations. Almost all antibody tests for the detection of *M. bovis* infections to date have suffered from a relative lack of Se in comparison to T-cell based assays because infected animals less frequently mount a significant humoral immune response to infection[41-43]. However, bovine data suggests that positive antibody test results can correlate with the presence of a visibly lesioned phenotype at *post mortem* carcass inspection and therefore likely reflect an animal which is not only infected, but also infectious to naïve animals and the environment that the animal is in[42]. A number of these heavily infected cattle will also not respond positively to the BOVIGAM™ test (an IGRA assay) due to T-cell anergy following prolonged antigenic stimulation[42,43]. Serological assays have additional technical advantages compared to IGRA tests in that they are simpler and are therefore quicker and cheaper to perform[42,43]. As well as this, because serological assays can be developed to include a small number of very specific antigen targets, the specificity of these assays can be greater than those seen for T-cell assays which frequently utilise antigen cocktails. These antigen cocktails may have shared epitopes with other mycobacteria which impedes the test specificity[44]. These factors mean that a combination of testing methods can be maximally effective in bringing *M. bovis* outbreaks under control, a strategy which has recently validated in an outbreak of *M. bovis* in cattle and goats[45,46]. Our previous study showed that the comparative PPD ELISA that we have developed identified the largest number of test-positive dogs of the serological assays tested with high Sp (set at 99% in that study). Here, we show that the same test retains a higher test Se than the other two serological assays with a median estimate of 32% (CrI 12-74%) compared to the DPP lateral flow VetTB assay and the Idexx *M. bovis* Ab ELISA which performed approximately half as well with median Se estimates of 17% and 18% respectively. This suggests that a combination of the IGRA and the comparative PPD ELISA might be the most efficient combination of two tests to use when investigating any similar outbreaks in the future though further research would be needed to confirm this.

Both of the assays shown to be the least sensitive in this analysis have contrastingly been shown to have much greater sensitivities in the species for which they were originally developed for use. For example, the DPP lateral flow VetTB assay performed with a sensitivity of 84.6% in a group of naturally and experimentally infected red deer (*Cervus elaphus*)[47] whilst the Idexx *M. bovis* Ab ELISA has a reported sensitivity of 63%[48]. The reasons for the differences in performance of these tests across the different species are not immediately obvious, however, the higher sensitivity of the comparative PPD ELISA which contains the greatest number of proteins suggests that the peptides included in the assays do not contain the immunodominant epitopes of *M. bovis* as recognised by the canine adaptive immune response. Therefore, the development of more sensitive serological assays will require greater interrogation of which antigen or antigen combinations are the most frequently recognised within *M. bovis* infected dogs.

An approach of combining serological testing subsequent to intradermal tuberculin injections may further increase the sensitivity of serological assays, as has been shown in other species[46,48], making this a useful area of focus for future research.

As would be predicted, all three of the serological assays demonstrated high specificities with median posterior Sp estimates of 76%, 77% and 89% respectively. These Sp values were all higher than the Sp estimate of the IGRA, estimated at 59% (CrI 44-80%). However, though lower than the serological assays, the Sp of the IGRA is within the range of values that would be expected for T-cell test assays, for example it is not significantly different to the specificity reported for the QuantiFERON^{TM}-TB Gold test in human populations with high tuberculosis burdens (70.8%; 95 % confidence interval 67.5–73.9 %)[36,39].

Because the data used in this study referred to a single outbreak we were able to estimate the prevalence of infection and we were also able to effectively estimate the positive and negative predictive values of the tests to give a clearer evaluation of the clinical utility of the tests.

The median posterior prevalence estimate produced from models two and three was 55%; this is similar to the percentage of animals that tested positive to at least one of our diagnostic

tests in the original outbreak investigation[12], however, this estimate was also linked to very a high degree of uncertainty with a 95% credibility interval of 5-96%.

As would be predicted, the IGRA was found to have both the highest estimated predictive values of the tests studied (median PPV 75%, median NPV 82%) whereas the serological assays had lower predictive values and performed comparably with one another. The posterior parameter estimates for the PPV and NPV produced from model three indicated that all of the tests we evaluated in this study met the criteria for being clinically informative by having values greater than 50%. This further demonstrates that these tests have the potential to be used in the diagnosis of canine *M. bovis* infections. Further work is needed to build on this analysis; the estimates for most of the modelled parameters sit within relatively wide credibility intervals due to the small size of the population of dogs tested (n=164), these can be improved by the inclusion of additional test data and would particularly benefit from data from dogs of known infection status.

8.5 Conclusion

This is the first study to analyse the test performance parameters of a number of diagnostic tests to detect *M. bovis* infections in domestic dogs. These results show that the IGRA test has the greatest Se, SP, PPV and NPV of the tests evaluated, it therefore has the greatest potential to be useful as a clinical diagnostic test. The three serological assays had markedly reduced Se estimates compared to the IGRA but despite this all three had predictive values which indicate they also have potential for use as diagnostic test. The comparative PPD ELISA assay that we developed was shown to be the best performing of the serological assays, possibly due to the inclusion of a broader pool of antigens. The development of serological tests for canine *M. bovis* infections could therefore benefit from the identification of specific immunodominant antigens in dogs and their inclusion in any novel assay development.

The use of a Bayesian latent class modelling approach was critical to this analysis given the lack of a gold standard test and that the true status of the individuals tested is unknown, however, it proved necessary to include informed priors to produce reliable posterior

parameter estimates. Future research evaluating the diagnostic test performance of these assays should include a greater number of animals, particularly those of known infection status, to establish greater credibility in the estimates produced. Additional work to assess the use of tests in combination with intradermal tuberculin injections may be beneficial to establishing the optimal testing strategies for future outbreak investigation and control.

8.6 References

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Chapter 9: Characterising Antigen-specific Cytokine Profiles for the Diagnosis of Canine *Mycobacterium bovis* Infections.

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Abstract

Tuberculosis (TB) has been the cause of considerable morbidity and mortality in the human population and zoonotic TB caused by *Mycobacterium (M). bovis* is an increasingly recognised pathogen of domestic companion animals. There are currently no validated *ante mortem* tests to diagnose TB in dogs. We previously measured mycobacterial-antigen specific interferon gamma (IFN-γ) as a marker of infection in a group of *M. bovis* exposed Foxhounds. The measurement of cytokines in addition to IFN-γ has been shown to improve TB diagnostic sensitivity in other species. Therefore we assessed the cytokine profile of antigen-stimulated peripheral blood mononuclear cells derived from *M. bovis* infected and uninfected (control) dogs to determine whether this might be of diagnostic potential for dogs. Supernatants from antigen stimulated cells were assessed using the Milliplex MAP Canine Cytokine Magnetic Bead multiplex assay. The pro-inflammatory cytokines, IL-8 and keratinocyte-derived chemokine (KC) concentrations were not significantly different between infected and control dogs whilst three cytokines; TNF-α, IL-6 and IL-10 showed potential for use as canine TB diagnostic markers along with IFN-γ. Future work should focus on the in-field utility of these findings to establish diagnostic sensitivity and specificity of these markers. The study also identified a number of similarities in the cytokine profile of TB affected dogs when compared

with more commonly studied species such as humans and cattle, indicating that further work studying the kinetics of canine TB infections could have translational benefits.

9.1 Introduction

Tuberculosis (TB) has been the cause of considerable morbidity and mortality in the human population for centuries, and remains the leading cause of death from any single infectious agent (*Mycobacterium (M.) tuberculosis*) worldwide [1–3]. Historically, the significance of this disease has resulted in extensive biomedical research interest and, due to the growing threat of drug resistance, TB is a major focus of collaborative global healthcare investment [4–8]. Meanwhile, TB in domestic companion animals receives relatively little research attention, despite being more common and clinically significant than previously thought [9–11]. In the UK, approximately 1% of all feline biopsies submitted for routine histopathological analysis show changes consistent with mycobacteriosis and a third of these contain Ziehl-Neelsen (ZN) positive organisms when stained, with morphology indicative of the presence of mycobacteria [10,12]. Culture results indicate that a third of these infections are tuberculous in cats [10,12], whilst equivalent data do not currently exist for dogs.

The various mycobacterial species that have been identified in companion animals, including dogs, can be grouped into the same two major categories as human mycobacterial disease; those belonging to the *Mycobacterium tuberculosis*-complex (MTBC) and the non-tuberculous mycobacteria (NTM, also referred to as ‘atypical mycobacteria’ or ‘mycobacteria other than tuberculosis’, MOTT) [11,13–19].

The MTBC consists of ten highly genetically related species of mycobacteria which are capable of causing TB in both man and other animals, and are some of the oldest recorded zoonotic diseases known to both human and veterinary medicine [20–26]. All member species of the complex share identical sequences across the 16s rRNA gene and 99.5% sequence homology across the remainder of the genome [27]. The most discriminating features between the species at the nucleotide level are genomic deletions, termed regions of difference (RD) [28–31]. The MTBC organisms which frequently infect dogs, *M. tuberculosis* and *M. bovis*, both

encode RD-1 [12,32–34]. This region is absent from *M. microti* and the vaccine strain; *M. bovis* bacille Calmette-Guérin (*M. bovis*-BCG) but present in all other MTBC organisms [28]. It has been shown to encode a variety of molecules which act as virulence factors such as the 6kDa early-secreted antigenic target (ESAT-6) and 10kDa culture filtrate protein (CFP-10) [35–37].

In companion animals, MTBC infections pose a potential zoonotic risk to their owners due to the close contact between owners and their pets and additionally these cases may act as potential sources of environmental contamination with mycobacteria such as *M. bovis*, a pathogen of major animal health significance in the UK as the causative agent of bovine tuberculosis (bTB) [38–41].

Although relatively rare, in contrast to human TB cases, all clinical canine TB cases diagnosed in an outbreak in a kennel displayed advanced renal pathology (reported in Chapter 5) [71]. Urine samples collected from these animals were shown to be culture positive for *M. bovis* which further increases the risk of environmental contamination and zoonotic transmission [71]. Pulmonary lesions in dogs have been reported to result from bacteria being inhaled, causing typical tubercle formation in the lungs and peri-bronchial lymph nodes [9,14]. Additionally, pulmonary disease is thought to occur secondarily to the putative haematogenous spread of bacteria from a site of inoculation elsewhere e.g. in the skin [12,14]. This generates a diffuse interstitial pattern of disease which eventually becomes bronchial and is clinically observable as progressive dyspnoea followed eventually by a productive cough [14,34]. Disseminated tuberculous disease has been reported to cause a range of clinical signs in dogs, including hepato-splenomegaly, pleural and pericardial effusions, generalised lymphadenopathy, weight loss and pyrexia [42].

With such variable reported clinical presentations and non-specific clinical signs, diagnosing canine MTBC infections rapidly and accurately is challenging. Mycobacterial culture conducted by the Animal and Plant Health Agency (APHA), is currently the recognised 'gold standard test' for the diagnosis of mycobacterial disease in UK companion animals; however, it has several limiting features. Firstly, when feline submissions were analysed it was shown to have a relatively poor sensitivity of less than 50% [9,34,43]. Secondly, it requires weeks from

the time that a contaminated tissue biopsy is submitted for results to be obtained *e.g.* the average culture time is 6-8 weeks for *M. bovis* [9,34,43]. During this time, if untreated, a patient will remain a potential source of infection to both its owner and the local environment.

As such, alternative diagnostic tests have been developed which aim to reduce the amount of time between clinical presentation and a definitive diagnosis being reached. Interferon-gamma (IFN-γ) release assays (IGRA) were developed on the principle of quantitatively evaluating IFN-γ production by peripherally circulating antigen specific effector T-memory cells upon *in vitro* stimulation, in order to aid the diagnosis of bTB in cattle [44–47]. These have subsequently been adapted to identify both active and latent TB in human patients with greater sensitivity and specificity than the previously utilised tuberculin skin test [48–50]. Where intra-dermal testing has been shown to be of unreliable clinical utility in the dog, the IGRA has been used successfully [14,46,71]. IGRAs have several advantages over other diagnostic techniques; they are significantly quicker at generating results than culture and are cheaper than many commercially available PCR and subsequent sequencing methods, they are also relatively non-invasive requiring only a single peripheral blood sample [46,49]. Unlike intra-dermal skin testing, they can be repeated if necessary as conducting the assay does not alter the systemic immune response [45,51,52].

Recently within the field of human diagnostics of mycobacterial diseases, and of TB in particular, there has been a marked increase in research focus on the identification of cytokine biomarkers for the diagnosis of both active and latent infections [53–58]. The goal of such assays is to cheaply, sensitively and rapidly identify infected and infectious individuals, in order to combat what remains one of the most incident infectious diseases of man [59]. Such studies have utilised multiplex cytokine assays, most commonly Luminex xMAP technologies, with promising results [55,60,61]. Studies of multiple assays have found cytokine concentrations and combinations of cytokines which can sensitively and specifically diagnose both active and latent TB in humans [53–58]. For example; the concentration of IL-2 within the IGRA supernatant can be combined with circulating levels of IFN-γ to more accurately discriminate between active and latent TB states [58,62]. Similar assays have also been used successfully in the study

of a limited number of companion animal diseases, including canine lymphoma, feline cystitis and feline mycobacterial infections (reported in Chapter 2) [63–66].

As the timely and accurate diagnosis of canine mycobacteriosis as well as the identification of the causative species is currently challenging, the aim of this study was to evaluate whether cytokine profiling using supernatants from stimulated peripheral blood mononuclear cells (PBMC) in dogs would demonstrate the same clinical utility as has been shown for humans. It was hypothesised that a number of cytokines would be differentially detectable in samples from dogs infected with *M. bovis* compared to uninfected controls. Since previous studies have shown that a proportion of TB affected dogs had antibodies specific for *M. bovis* antigens (Chapters 5 and 7), this study analysed whether the cytokine profile also differed in these individuals.

9.2 Materials and Methods

This study was conducted with prospective approval from the School of Veterinary Medicine Ethical Review Committee at the University of Edinburgh and all relevant guidelines and regulations were adhered to throughout.

9.2.1 Sample Collection

Cell based IGRA assays for the 164 kennel hounds which were tested as part of an outbreak of TB caused by *M. bovis* infection were conducted as previously described (Chapter 5) [71]. The stored supernatants from PBMC stimulated by purified protein derived from *M. bovis* (PPDB) or a combined peptide cocktail of ESAT-6 and CFP-10 antigens were compared to supernatants from unstimulated PBMC. Samples from 60 hounds were selected for analysis.

The hounds were classified into one of three groups based on the combined outcome of the diagnostic tests performed previously. As detailed in O'Halloran *et al.* (2018) [71], dogs with significant mycobacterial antigen specific IFN-γ responses were classed as IGRA positive. In addition to the IGRA, the sera were tested with the DPP lateral flow assay, the Idexx *M. bovis*

Ab ELISA and a comparative PPD antibody ELISA [72]. Here, these results were combined to class hounds as either seropositive if they were shown to have responded positively in at least one of the antibody tests, or seronegative if they did not.

The previous test results were used to establish the three groups of dogs analysed in this current study. Firstly, dogs which were test-negative by IGRA and all three serological assays on two occasions 30 days apart (uninfected hounds, control group). This group was used as the control group against which the cytokine responses of the other groups could be compared. Secondly, dogs which were found to be positive to the IGRA test but which were negative to all of the serological assays (IGRA+, seronegative) and thirdly, dogs which were IGRA test positive and positive to at least one serological assay (IGRA+, seropositive). No dogs in this study were test positive on the serological assays and test negative by IGRA.

9.2.2 Cytokine and Chemokine Measurements

Cytokine and chemokine concentrations were measured in supernatant samples diluted 1:2 in RPMI 1640 cell culture media (Gibco, Thermo Fisher Scientific, UK) using a commercial, canine specific, antibody-coated microsphere-based multiplex cytokine immunoassay shown to be able to quantify 13 cytokines contemporaneously using 25µL of each sample (CYTOMAG-90K MILLIPLEX MAP Canine Cytokine/Chemokine Magnetic Bead Panel, Premix 19 Plex kit, MERCK Millipore Corporation, Billerica, MA, USA).

The following cytokines were measured: granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, interleukin (IL)-2, IL-6, IL-7, IL-8, IL-10, IL-15, interferon gamma-induced protein (IP)-10, IL-18, keratinocyte-derived chemokine-like protein (KC-like), monocyte chemoattractant protein 1 (MCP-1, also known as CCL2) and tumour necrosis factor (TNF)-α.

All reagents as well as the supernatant samples were brought to room temperature. The pre-mixed bead solution was sonicated for 30 seconds and then vortexed for a minute. Quality Control vials 1 and 2 (CCTYOMAG-90K Kit) were each reconstituted with 250µL of deionised water. The standard curve panel was produced by conducting a seven step 4-fold dilution

series of the Canine Cytokine Panel Standard in Assay Buffer (CCTYOMAG-90K Kit) following reconstitution in 250µL of deionised water. Each assay plate was initially coated with 200µL/well of Assay Buffer which was left for ten minutes on a plate shaker prior to being removed. An aliquot of 25µL of kit Standard, Control or supernatant sample was added to each well as appropriate; all samples were assayed in duplicate and mean values analysed. An additional 25µL of Assay Buffer was added to the 0pg/mL standard (background) wells and to each well containing test sample whilst the same volume of Assay Buffer was added to the Standard and Control wells. Finally, 25µL of the pre-mixed bead solution was added to each well before the plate was covered with aluminium foil and incubated overnight (at least 16 hours) at 4^{o}C on a plate shaker.

The following day, the contents of each well were removed and each well was washed twice with 200µL of Wash Buffer. The Detection Antibody solution (CCTYOMAG-90K Kit) was allowed to warm to room temperature before 25µL was added to each well. The plates were sealed, covered with aluminium foil and incubated at room temperature on a plate shaker for an hour before 25µL of streptavidin-phycoerythrin (CCTYOMAG-90K Kit) was then added to each well. The plate again was incubated at room temperature on a plate shaker for an additional 30 minutes. The contents of each well was removed and each well was washed twice with 200µL of Wash Buffer.

The plates were read with a multiplex plate reader and companion software (Luminex 200). All cytokine and chemokine concentrations were calculated by the software prior to statistical analysis and the results are reported in pg/mL.

9.2.3 Statistical Analysis

Statistical analysis was performed using commercially available statistical software (GraphPad Prism 7.0). Mean concentration values of duplicate samples for each condition were doubled prior to analysis to allow for the dilution factor. For the purposes of statistical analysis, values that fell below the limit of detection of the assay were assigned a concentration of 0 pg/mL.

The amount of antigen specific cytokine was determined by subtracting the concentration in the unstimulated cells from both the PPBD stimulated and ESAT-6/CFP-10 stimulated wells. The 60 dogs tested were divided into three groups based on their previous test results; IGRA negative and seronegative (controls, n=22), IGRA+ seronegative (n=22) or IGRA+ seropositive (n=16).

A D'Agostino & Pearson omnibus test was used to assess the data. This showed that the data did not conform to a Gaussian distribution and therefore non-parametric tests were used for the analysis; Kruskal-Wallis tests were used to determine if there were significant differences between the three groups of dogs. If this was significant then pairwise Mann-Whitney tests were performed to determine which groups of dogs were significantly different. The two sets of data, from the PPDB stimulated samples and the ESAT-6/CFP-10 samples were analysed independently. Statistical significance for all tests was set a $p<0.05$.

9.3 Results

Cytokine concentrations were measured in the supernatants from antigen-stimulated PBMC from three groups of dogs; IGRA negative seronegative (uninfected controls), IGRA+ seronegative and IGRA+ seropositive. The median concentration and associated 95% confidence intervals (CI) are given for all cytokines separated into each of the three groups of dogs; Table 9.1 shows the cytokine concentrations measured from the supernatant of PPDB stimulated cells and Table 9.2 shows the equivalent values present within the supernatants of ESAT-6/CFP-10 stimulated cells. The concentrations of cytokines that were found to be significantly different between two or more of the three groups of dogs are shown in Figure 9.1.

Table 9.1: Cytokine concentrations measured from the supernatant of PPDB stimulated cells; values are given as the median and associated 95% confidence interval (CI). All concentrations are presented in pg/mL.

	IGRA negative & Seronegative (controls)			IGRA positive & Seronegative			IGRA positive & Seropositive		
	Median	Upper 95% CI	Lower 95% CI	Median	Upper 95% CI	Lower 95% CI	Median	Upper 95% CI	Lower 95% CI
GM-CSF	0.00	0.00	0.00	0.00	158.50	0.00	0.00	412.60	0.00
IFN-γ	0.33	9.10	0.00	252.30	503.20	204.30	13.65	185.90	0.00
IL-2	0.00	0.00	0.00	0.00	1.05	0.00	0.00	0.00	0.00
IL-6	0.00	33.45	0.00	34.39	110.40	6.92	25.45	61.01	8.42
IL-7	0.00	0.00	0.00	0.00	2.63	0.00	0.00	2.05	0.00
IL-8	5939.00	19115.00	0.00	29215.00	52803.00	4135.00	14607.00	59728.00	0.00
IL-15	0.00	0.58	0.00	0.00	7.74	0.00	0.00	7.60	0.00
IP-10	0.00	0.00	0.00	0.00	1.72	0.00	0.00	0.00	0.00
KC-like	521.90	1220.00	0.00	649.10	814.50	177.10	1430.00	1764.00	434.20
IL-10	5.49	20.45	0.00	24.93	81.44	0.00	30.05	120.30	8.93
IL-18	0.00	0.00	0.00	0.00	1.05	0.00	0.00	0.43	0.00
MCP-1	0.00	1633.00	0.00	0.00	4154.00	1081.00	3087.75	82857.00	2077.00
TNFα	3.11	8.68	0.00	39.77	118.30	29.81	36.90	139.00	1.94

Table 9.2: Cytokine concentrations measured from the supernatant of ESAT-6/CFP-10 stimulated cells; values are given as the median and associated 95% confidence interval (CI). All concentrations are presented in pg/mL.

	IGRA negative & Seronegative (controls)			IGRA positive & Seronegative			IGRA positive & Seropositive		
	Median	Upper 95% CI	Lower 95% CI	Median	Upper 95% CI	Lower 95% CI	Median	Upper 95% CI	Lower 95% CI
GM-CSF	0.00	0.00	0.00	0.00	11.22	0.00	0.00	60.78	0.00
IFN-γ	1.93	10.83	0.00	102.70	330.40	9.10	6.54	81.38	0.00
IL-2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
IL-6	0.93	17.35	0.00	13.27	31.39	0.00	2.87	40.09	0.00
IL-7	0.00	0.00	0.00	0.00	0.32	0.00	0.00	0.50	0.00
IL-8	2715.00	21803.00	0.00	9074.00	22250.00	4.74	7485.00	45612.00	0.00
IL-15	0.00	3.99	0.00	0.00	2.79	0.00	0.00	7.08	0.00
IP-10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
KC-like	330.20	1229.00	0.00	206.70	696.10	32.22	371.10	1003.00	0.00
IL-10	9.13	36.32	0.00	14.44	26.90	0.00	7.01	29.89	0.00
IL-18	0.00	0.00	0.00	0.00	0.97	0.00	0.00	0.50	0.00
MCP-1	0.00	0.00	0.00	256.00	2163.00	0.00	4659.50	68135.00	3721.30
TNFα	5.55	30.90	0.00	24.24	42.15	14.59	0.00	46.71	0.00

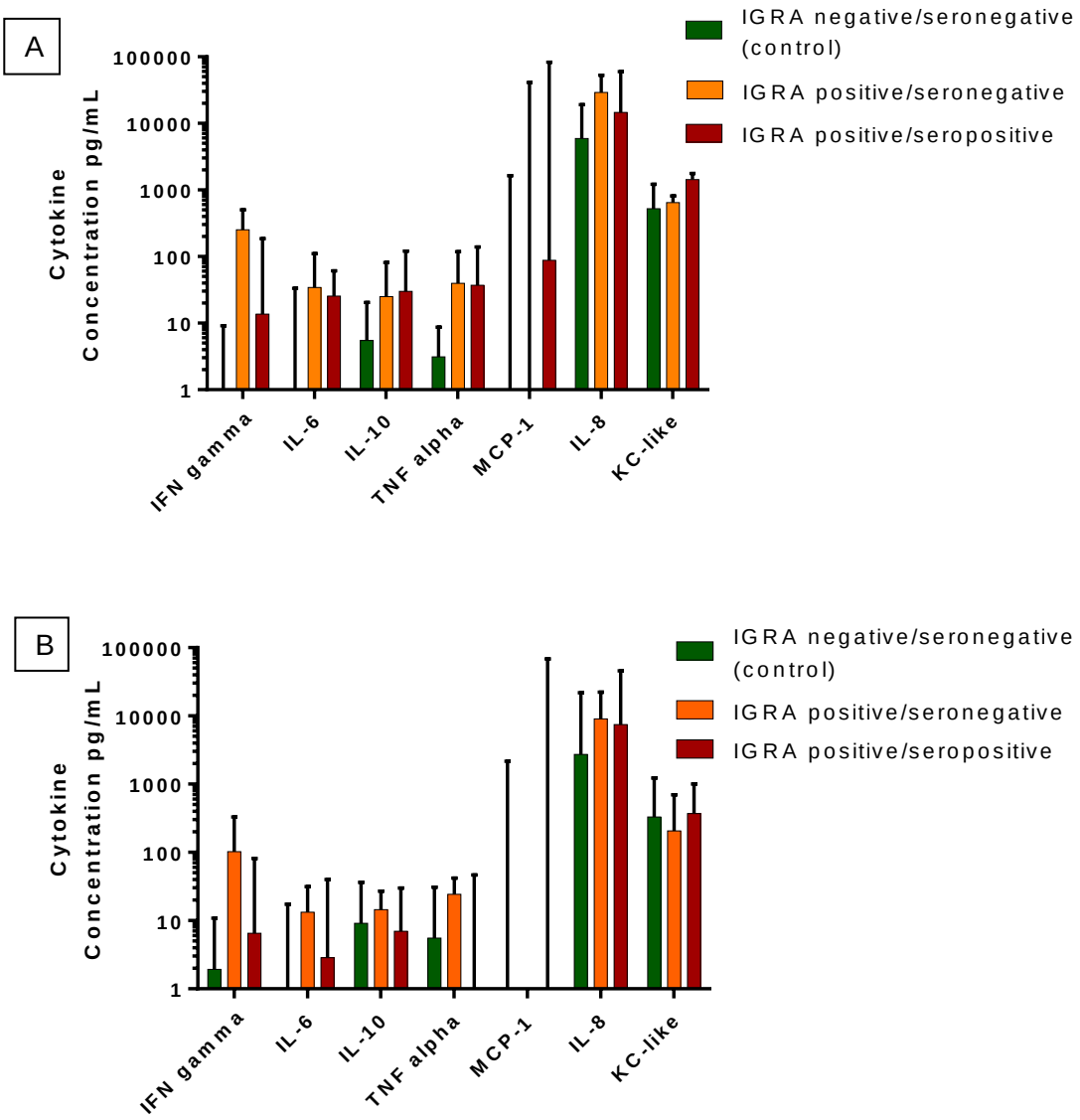


Figure 9.1: Cytokines identified in the supernatant of peripheral blood mononuclear cells stimulated with either PPDB (A), or ESAT-6/CFP-10 (B). Cells were taken from dogs previously diagnosed as uninfected (green), *M. bovis* IGRA+ and seronegative (orange) or *M. bovis* IGRA+ and seropositive (red). Data are displayed as the median concentration for the group with error bars indicting the 95% confidence interval.

Cells stimulated with either PPDB or ESAT-6/CFP-10 generated significantly different concentrations of IFN-γ between all three groups of dogs ($p<0.0001$). The IGRA+ seronegative animals were found to produce significantly greater concentrations of IFN-γ from PBMC stimulated with both PPDB and ESAT-6/CFP-10 antigens when compared to the other groups of dogs ($p<0.001$). Similarly, the IGRA+ seropositive dogs had PBMC which produced

significantly higher concentrations of IFN-γ in response to both antigens than the control group ($p<0.001$).

In response to PPDB stimulation, cells from both groups of IGRA+ dogs produced higher concentrations of IL-6 than the IGRA negative group (Figure 9.1 A, $p=0.002$). There was no significant difference between the concentration of IL-6 produced by PBMC from the IGRA+ seronegative dogs and the IGRA+ seropositive dogs (Figure 9.1 A, $p=0.136$). However, in response to ESAT-6/CFP-10 stimulation, cells from both groups of IGRA+ dogs produced a greater concentration of IL-6 than the IGRA negative control group (Figure 9.1b, $p=0.007$) whilst the PBMC of IGRA+ seropositive dogs produced significantly less IL-6 than those taken from IGRA+ seronegative dogs (Figure 9.1b, $p=0.03$).

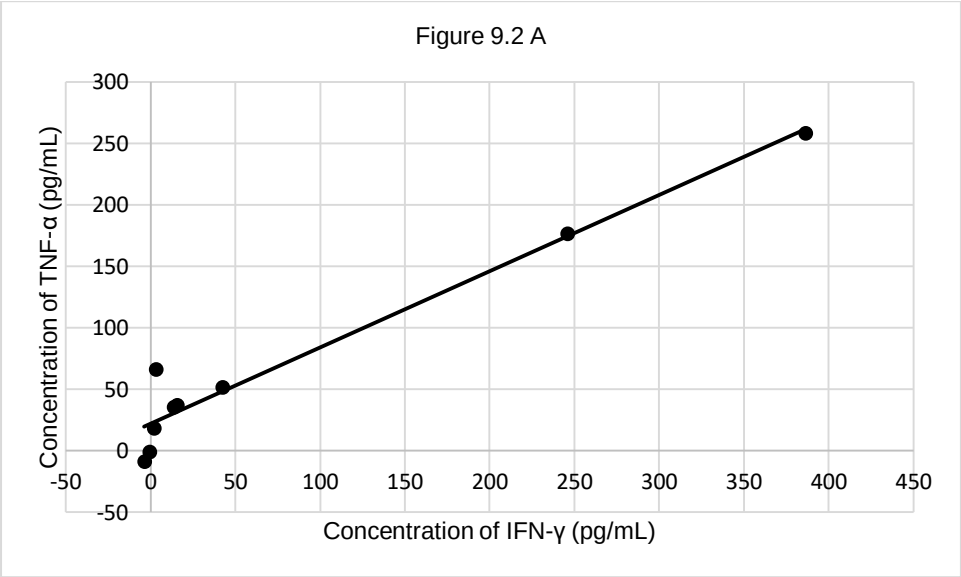
The cytokine IL-10 was found to be secreted to higher concentrations by cells from IGRA+ dogs than from IGRA negative dogs in response to both antigens (Figure 9.1, $p<0.001$). Whilst there was no difference between the concentrations of IL-10 produced by PBMC from IGRA+ seronegative and IGRA+ seropositive dog PBMC stimulated with PPDB (Figure 9.1 A). There was significantly less IL-10 produced by cells from IGRA+ seropositive dogs in response to ESAT-6/CFP-10 than by IGRA+ seronegative dogs, but the former was not then significantly different to the concentration produced by cells from control dogs (Figure 9.1 B, $p=0.06$).

The concentration of TNF-α was significantly higher in the supernatant of cells from IGRA+ seronegative dogs compared to the control dogs in response to both PPDB and ESAT-6/CFP-10 antigens (Figure 9.1, $p=0.01$). The cells from IGRA+ seropositive dogs produced an equivalent concentration of TNF-α as the IGRA+ seronegative dogs when stimulated with PPDB but did not produce consistently detectable concentrations of TNF-α when cells were stimulated with ESAT-6/CFP-10 (Figure 9.1).

The cytokines IL-8 and KC-like were found to be produced in response to both antigens by cells from all three groups of dogs, however, no significant differences were found between any of the groups (Figure 9.1, $p=0.3293$, $p=0.8698$ respectively).

No significant difference was found in the concentration of MCP-1 produced under any of the conditions or groups (p=0.5). However, three dogs within the control group had very high levels (>10,000pg/mL) of MCP-1 in response to ESAT-6/CFP-10 stimulation which skewed these data considerably.

Based on previous study findings of good agreement between the amount of antigen specific TNF-α and IFN-γ in IGRA+ dogs (Chapter 7, Section 7.3.1) [71,72], the concentration of TNF-α was compared to the amount of IFN-γ in the same supernatants. There was near perfect positive correlation between the two cytokines in the cells stimulated with PPDB from all three groups of dogs (R^2=0.95, Figure 9.2 A) but only weak positive correlation was seen in cells stimulated with ESAT-6/CFP-10 (R^2=0.34, Figure 9.2 B).



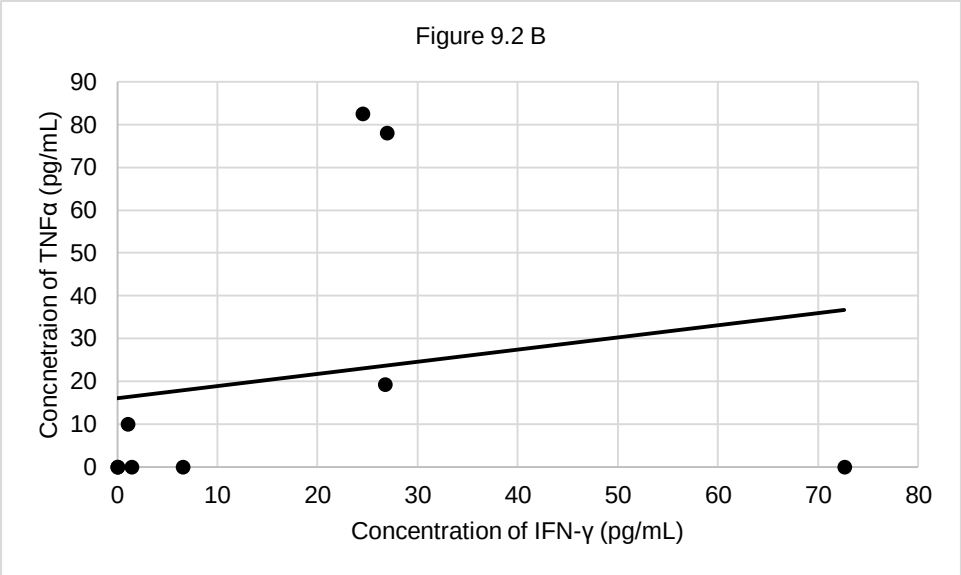


Figure 9.2: The concentration of TNF-α plotted against the concentration of IFN-γ in the supernatants of peripheral blood mononuclear cells obtained from all 60 dogs included in this study stimulated with either PPDB (9.2 A) or ESAT-6/CFP-10 (9.2 B). A line of best fit plotted on both figures shows a strong positive correlation for PPDB stimulated samples (R^2=0.95) whilst there is only weak association for cells stimulated with ESAT-6/CFP-10 (R^2=0.34).

The cytokines GM-CSF, IL-2, IL-7, IL-15, IP-10 and IL-18 were not found at concentrations above the limit of detection in any of the samples analysed (Table 9.1, Table 9.2).

9.4 Discussion

Tuberculosis due to *M. bovis* in dogs represents a zoonotic risk for owners but the diagnostic options are currently limited [71,72]. Whilst the concentration of the cytokine IFN-γ produced by PBMC or whole blood stimulated with mycobacterial antigens has long been used to diagnose infections in a number of species, recent studies in human patients infected with *M. tuberculosis* and cattle infected with *M. bovis* have shown that a number of other cytokines are produced under these conditions and can be used to infer more information about the state of a patient's infection such as whether or not there is active disease, or to estimate prognosis [73-75]. The aim of this study was to analyse the concentration of multiple cytokines produced by canine PBMC in response to mycobacterial antigens from dogs previously diagnosed as infected or uninfected with *M. bovis.* The findings were related to the IGRA and serological status of dogs in relation to anti-mycobacterial antibodies measured as part of previous studies (Chapter 5 and Chapter 7) [71,72]. All dogs were adult working Foxhounds co-habitant in the same kennel at the time of an *M. bovis* outbreak.

Samples from 60 dogs were analysed in three groups; IGRA negative seronegative (controls, n=22), IGRA+ seronegative (n=22) or IGRA+ seropositive (n=16). These samples were chosen based on the results of previous tests to keep the size of each group proportionally similar to those seen when the entire pack was tested [71,72]. We previously showed that more than half of the kennel hounds were positive by IGRA test, here 63% of dogs were IGRA+, whilst approximately a third of the hounds were previously shown to have anti-mycobacterial antibodies and 37% of dogs included in this study were seropositive [71,72]. Initial analysis of the concentration of IFN-γ using this assay matched the results found previously when the supernatants were tested by conventional ELISA, validating the classification of dogs in this study.

Notably, the level of antigen specific IFN-γ and IL-6 detected here showed a broadly similar pattern of secretion to each other. The concentrations of both cytokines were lower in the supernatant of cells stimulated with ESAT-6/CFP-10 than with PPDB. There are several

reasons that this may be the case; firstly, ESAT-6 and CFP-10 proteins are released from *M. bovis* via the ESX-1/typeVII secretion system that is not always active [76-78]. This consistently gives rise to a number of animals that are infected with *M. bovis* but lack an antigen-specific response to these proteins. In experimentally infected cattle, studies show this to be approximately 18% and one feline study found it to be the case in two of ten (20%) *M. bovis* infected cats [46,76-78]. Alternatively, these proteins may not be immunogenic to every animal due to genetic differences such as polymorphisms in MHC class II genes, or more subtle differences in antigen processing and presentation. This would in turn lower the median value measured across the group. Furthermore, PPDB contains a great number of peptides, all of which may provide stimulation to primed T-cells compared to just two proteins in the ESAT-6/CFP-10 cocktail and this may lead to the secretion of a lower overall concentration of IFN-γ in these samples.

The concentration of IFN-γ and IL-6 was also found to be lower in the supernatants derived from seropositive dogs compared to seronegative dogs in response to both antigens (PPDB and ESAT-6/CFP-10). As IFN-γ is secreted by, amongst other cells, activated T-cells and IL-6 is released from blood monocytes and tissue macrophages, this likely reflects the activation of a Th-1 immune response classically triggered by intracellular pathogens such as mycobacteria [79]. The decreased levels seen in seropositive animals may indicate that these dogs are moving towards a weaker Th-1 but stronger Th-2 biased immune response, which in human TB patients has been shown to be associated with active disease [80]. Quantifying cytokines more typically associated with a stronger Th-2 response such as IL-4 and IL-13 may help to identify dogs at risk of developing clinical signs of TB so that they can be more quickly targeted for intervention.

The cytokine IL-10 is a major immunoregulatory factor, particularly in the context of TB, and in humans and cattle has been shown a negative feedback effect on the production of IFN-γ with the effect of limiting the pro-inflammatory response to avoid excessive immune-mediated pathology [81-85]. It was therefore unexpected to see higher levels produced by PBMC from IGRA+ dogs than cells from dogs that were IGRA negative. However, it has recently been

demonstrated that Th-1 $CD4^+$ T-cells will secrete both IFN-γ and IL-10 following prolonged antigenic stimulation [81-85]. Given the pattern of detection for both of these cytokines shown herein this suggests significant antigen specific T-cell responses are ongoing in the IGRA+ group of dogs.

No IL-2, IL-7 or IL-15 was recovered from any of the supernatants in this study despite human and bovine studies suggesting that IL-2 concentrations can be indicative of more severe disease phenotypes [74,75]. These three cytokines are a closely related group with a shared gamma chain (γ_c) structure allowing them to share some cellular receptors and regulate immune cell differentiation [86-88]. In particular, they can modulate naïve, effector, and memory $CD4^+$ T-cell function and IL-2 was initially identified as an autocrine cytokine necessary for the *in vitro* expansion of T-cells [86-88]. In the context of the IGRA, IL-2 is detectable in bovine blood samples where antigen specific T-cells are expanding eight hours after antigen is added [74]. In our study, we assayed the supernatants after four days of incubation and so any of the three γ_c-cytokines produced will likely have bound back onto cell receptors to induce continued activation/proliferation and so not be available for detection in the supernatant. Future studies examining the kinetics of cytokine production and secretion in this assay would be beneficial to determine the time point of optimum test sensitivity.

No GM-CSF was consistently detected in any of the canine samples we analysed. A recent study of health care workers with exposure to patients with *M. tuberculosis* infections found no significant differences in the quantity of antigen specific GM-CSF produced by stimulated whole blood samples until exposure was prolonged (more than five years) where it was shown to be protective against active disease in these individuals [89]. A number of *in vitro* studies have shown that GM-CSF has a limiting effect on *M. tuberculosis* replication and study of human PBMC demonstrated that activated NK cells improve γδ T-cell proliferation by secreting GM-CSF, along with other cytokines, when stimulated with *M. tuberculosis* antigens [90,91]. Given these previous studies, it was unexpected that little or no GM-CSF would be identified in our samples; however, very little is known about the biology of canine NK cells, and an exact definition of a canine NK cell is lacking from the literature [92]. The lack of antigen specific GM-

CSF in this study may further indicate that canine NK cells display different behaviour or functionality in the context of tuberculosis than in other species such as humans and mice. Alternatively, this finding may reflect an earlier stage of infection in these dogs compared to human studies where GM-CSF was only identified after years of exposure; whereas all of the canine samples were taken within three months of the first clinical case being diagnosed which may be too soon in a chronic disease process to identify this marker of protection.

The pro-inflammatory cytokines IL-8 and KC-like were present at equivalent concentrations in all groups that we tested. Our research (Chapter 3) has previously shown that whilst these cytokines are present in higher concentrations in the sera of mycobacteria infected cats when compared to controls, this increase is non-specific and is found in cats with non-infectious health problems [93]. Therefore, the cytokines detected in this study may similarly reflect a non-specific response.

As we have previously shown for a small number of dogs (Chapter 7) [72], the cytokine TNF-α showed a similar pattern of secretion to IFN-γ with a very strong correlated response in the supernatant of cells stimulated with PPDB. This suggests that within the assay there is reciprocal activation of canine monocytes by IFN-γ likely produced by ($CD4^+$) T-cells and subsequent agonism of TNF-receptors expressed by T-cells. Although this process may be dependent on other cytokines produced from other PBMC subsets, this may facilitate the development of an *in vitro* co-culture system for studying the canine immune response to mycobacteria more closely in future studies.

In conclusion, this study is the first to analyse the cytokine production profiles of canine PBMC derived from *M. bovis* infected or uninfected dogs. It confirms that measuring antigen specific IFN-γ production is a robust mechanism for the classification of the infection status of dogs whilst it is the first suggesting that determining antigen specific concentrations of the cytokines IL-6, IL-10 and TNFα may have potential the be developed into sensitive diagnostic tests.

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Chapter 10: Canine Tuberculosis - A review of 19 new UK cases and 993 global historical cases.

Abstract

Canine tuberculosis has been recognised as a disease of dogs since shortly after the identification of *M. tuberculosis* itself at the end of the 19th century. The intimate relationship that has been built between humans and dogs during their millennia of domestication means that the zoonotic and reverse zoonotic transmission of diseases to and from domestic canines, including tuberculosis, if of significant public health significance.

This analysis considered 1012 canine tuberculosis cases including 19 previously unreported cases and 993 from the historical literature. This study found that all cases were caused by infections with one of three MTBC organisms, *M. tuberculosis, M. bovis* or *M. mircroti.* This study found that during the last decade (2009-2019), the pattern of infections has changed; with a relative decline in the significance of *M. tuberculosis* as compared with *M. bovis* and the first documented canine infections with *M. microti.*

The diagnosis of canine tuberculosis remains challenging as all of the three most frequently used tests; culture, interferon gamma release assay (IGRA) and PCR have unproven sensitivities with respect to dogs. Specialist culture remains the gold standard method to establish a diagnosis but the time required (weeks to months) means that an adjunctive test method such as and IGRA and/or PCR test may be necessary whilst culture results are pending.

All cases conformed to one of four clinical groups based on the presenting signs; primary pulmonary, primary abdominal, disseminated or miscellaneous. For all presentations of cases, other than dogs with disseminated disease, treatment with triple combination antimicrobial therapy lead to good clinical outcomes but the treatment of dogs with *M. tuberculosis* remains against advice due to the public health risk.

10.1 Introduction

Clinical tuberculosis has been documented in a variety of mammalian species as the result of infection with members of the *Mycobacterium tuberculosis*-complex (MTBC) group of mycobacteria (Brougham *et al.* 2013). This group of bacteria are non-motile, non-spore forming, intracellular, Gram-positive aerobic bacilli that are characteristically acid-alcohol fast when visualised by special stains, typically Ziehl-Neelsen staining, of tissue sections (Deforeges *et al.* 2014).

The MTBC comprises phylogenetically related mycobcateria that share remarkable levels of genomic nucleotide sequence homology and share many identical gene sequences (Warner *et al.* 2013). The complex includes *Mycobacterium (M.) tuberculosis*, *M. bovis*, *M. bovis* bacile Calmette–Guérin *(M. bovis*-BCG), *M. microti*, *M. canettii*, *M. africanum*, *M. pinnipedii*, *M. caprae*, *M. suricattae* and *M. mungi,* with all but the latter two having been reported as causing disease in humans (Pinsky *et al.* 2008; Warner *et al.* 2013).

The domestic dog (*Canis lupus familiaris*) has a unique affiliation with human society that has developed since their domestication *circa* 40,000 years ago (Horard *et al.* 2014). This relationship has placed the dog in a position to be both the recipient and origin of transmission for many human pathogens including the mycobacteria of the MTBC. Clinical tuberculosis caused by the human adapted *M. tuberculosis* was first confirmed in a dog in 1892, merely seven years after the organism was first identified by Robert Koch (Moore, 1928; Gradman, 2006). Canine tuberculosis received significant attention in the UK in the late 1920s and early 1930s when it was first shown that companion dogs can act as both spill-over hosts and as a sentinel species for human infections (Moore, 1928; Thayer *et al.* 1930; Adams and Vokwald *et al.* 1932; Steinbach and Deskowitz, 1933). Throughout the 1930's and beyond, dogs were used as an experimental model for human tuberculosis and canine serum was even explored as a potential therapeutic for patients in the pre-antimicrobial era (Beretta, 1891; McFadyean, 1891; Manwaring, 1913; Adams and Volkard, 1932)

In the last 75 years, the incidence of tuberculosis in the human population throughout the UK as declined by 90% and the mortality rate has fallen by 98% (Glaziou *et al.* 2018). Throughout the same period, reports of canine tuberculosis in the literature saw a similarly dramatic decline and began to reflect individual and sporadically occurring canine cases whilst reports of feline mycobacteriosis became largely limited to incidental findings during *post mortem* surveys (Dodd, 1952, Cordes *et al.* 1963; Birn *et al.* 1965).

In recent years, however, there appears to have been an anecdotal increase in the number of diagnoses of canine tuberculosis being made in the UK. Since December 2016, the author is aware of more than 120 cases; including one major outbreak in a kennel of Foxhounds (O'Halloran *et al.* 2018). There has also been increasing awareness in the last decade of the significance of mycobacterial infections in cats in the UK (Gunn-Moore *et al.* 2011; Gunn-Moore *et al.* 2013). Meanwhile, the incidence of bovine tuberculosis (caused by infection with *M. bovis*) has also been increasing. It therefore seems that canine tuberculosis cases are likely to re-emerge as a clinical entity in the UK but this disease remains poorly characterised.

It is the purpose of this study to review a series of both previously unreported cases of canine MTBC infections as well as those previously presented in the literature. This study discusses their clinical presentations, the challenge of diagnosis, potential treatment options as well as the zoonotic and reverse zoonotic potential of these bacteria.

10.2 Materials and Methods

Data relevant to the cases discussed in this study were obtained by literature review using online resources (PubMed, Web of Knowledge, CAB Abstracts, Medline and Google Scholar) with the search terms 'dog or dogs or canine', 'tuberculosis or TB and mycobacteri*'. Cases were only included if infection with MTBC organisms were demonstrated *i.e.* cases of infection with other mycobacterial species were omitted.

In instances of previously unpublished cases, all were managed under the supervision of the author. The primary care veterinary surgeon for each case was contacted and asked to provide clinically relevant data and to obtain consent from the owners for the data to be used in this

study. The study was conducted with ethical approval from the University of Edinburgh, Veterinary Ethical Review Committee.

For each case where data were available signalment data including; location (country), breed, age at diagnosis, sex and neuter status were recorded. The method/s by which each diagnosis was made and the causative organism was recorded to the species level wherever this was available; however, in some cases it was only possible to diagnose infections to within the MTBC. Any treatment given to cases (where this was done) and the outcome was also recorded.

10.3 Results

A total of 1012 instances of canine tuberculosis were examined in this study; 19 cases were treated under the supervision of the author and a further 993 cases were identified by reference to previously published studies. The major details of the cases are summarised in Table 10.1. The amount of detail pertaining to the clinical particulars of each case presented in historical publications was variable. Therefore, where possible data are presented quantitatively but the cases are sometimes referred to qualitatively.

Table 10.1: Key details of the canine tuberculosis cases included in this study. No,: number of cases in each publications; Country: country of residence of the dogs at the time of the diagnosis; Year: year that the diagnosis was made; Age: age at the time of the diagnosis, Method: method by which the diagnosis was made and the species confirmed. NR: details not recorded/detailed in publication.

No.	Country	Year	Age	Sex	Breed	Organism	Method	Source
1	UK	2018	7	MN	Cross-breed	*M. bovis*	PCR	This study
1	UK	2017	9	FN	German Shepherd	*M. bovis*	PCR	This study
1	UK	2017	9	FE	Labrador	*M. bovis*	PCR	This study
1	UK	2018	3	MN	Cavalier King Charles Spaniel	*M. bovis*	PCR	This study
1	UK	2019	6	MN	Beagle	MTBC	PCR	This study
1	UK	2018	4	ME	Springer Spaniel	*M. microti*	PCR	This study
1	UK	2016	1	FE	Staffordshire Bull Terrier	MTBC	PCR	This study
1	UK	2019	4	MN	Greyhound	*M. bovis*	PCR & culture	This study
1	UK	2015	4	FN	Staffordshire Bull Terrier	MTBC	IGRA	This study
1	UK	2013	2	FN	Italian Spinone	*M. bovis*	IGRA + history	This study
1	UK	2013	5	ME	Italian Spinone	*M. bovis*	Culture	This study
1	UK	2015	7	FN	Catalunyan Sheepdog	*M. microti*	PCR	This study
1	UK	2015	4	FN	Mixed	*M. tuberculosis*	IGRA + history	This study
1	UK	2012	6	FE	Jack Russell Terrier	*M. bovis*	Culture	This study
1	UK	2013	6	FN	Lurcher	*M. bovis*	IGRA + history	This study
1	UK	2013	2	ME	Lurcher	*M. bovis*	IGRA + history	This study

1	UK	2009	7	ME	Patterdale Terrier	*M. bovis*	Culture	This study
1	UK	2008	6	ME	Jack Russell Terrier	*M. bovis*	Culture	This study
1	UK	2007	3	ME	Mixed Terrier	*M. bovis*	Culture	This study
1	UK	2014	4	MN	Mixed Breed	*M. tuberculosis*	Culture	Englemann *et al.* 2014
97	UK	2017	2-4 years	Mixed	Foxhound	*M. bovis*	IGRA, PCR & culture	O'Halloran *et al.* 2018
1	UK	2004	6	ME	Border Collie	*M. bovis*	Culture	Ellis *et al.* 2006
1	UK	2008	NR	NR	NR	*M. bovis*	Culture	Shrikrishna *et al.* 2009
7	UK	2004-2010	NR	NR	NR	*M. bovis*	Culture	Broughan *et al.* 2013
1	Eire	2004	3	ME	Fox Terrier	*M. bovis*	Culture	Bauer *et al.* 2004
1	France	2013	NR	NR	NR	*M. microti*	Culture	Michelet *et al.* 2015
1	France	2011	NR	NR	NR	*M. microti*	Culture	Michelet *et al.* 2015
1	Switzerland	2011	7	MN	Podengo Iberico	*M. tuberculosis*	PCR	Posthaus *et al.* 2011
1	France	2004	NR	NR	NR	*M. microti*	Culture	Michelet *et al.* 2015
1	France	2002	4	ME	Beauceron	*M. microti*	Culture	Deforges *et al.* 2004
1	South Africa	2008	NR	ME	Maltese cross	*M. tuberculosis*	Culture	Parsons *et al.* 2008
3	South Africa	2012	Mixed	Mixed	Mixed	*M. tuberculosis*	Culture	Parsons *et al.* 2012
1	Norway	1982	NR	NR	NR	*M. tuberculosis*	Culture	Saxegaard, 1982
1	USA	2004	3.5	ME	Yorkshire Terrier	*M. tuberculosis*	Culture	Erwin *et al.* 2004

37	USA	2004-2011	Mixed	Mixed	Mixed	*M. bovis*	Culture	Thacker *et al.* 2013
1	Belgium	1991	6	ME	Red Setter/Cocker Spaniel cross	*M. tuberculosis*	Culture	Clercx *et al.* 1992
5	USA	1987	Mixed	Mixed	Basset Hound	*M. tuberculosis*	Culture	Carpenter *et al.* 1988
1	USA	1982	6	ME	Cross breed	*M. tuberculosis*	Culture	Ferber *et al.* 1983
1	UK	1964	5	FE	Yorkshire Terrier	*M. tuberculosis*	Culture	Birn *et al.* 1965
1	USA	2004	3.5	MN	Yorkshire Terrier	*M. tuberculosis*	Culture	Hackendahl *et al.* 2004
1	USA	1986	0.5	FE	Mixed	*M. tuberculosis*	Culture	Foster *et al.* 1986
1	Germany	2007	6	NR	Golden Retriever	*M. tuberculosis*	Culture & PCR	Deppenmeier *et al.* 2007
1	Czech Republic	2011	5	FE	Doberman	*M. tuberculosis*	Culture & PCR	Moravkova *et al.* 2011
1	New Zealand	2000	8	ME	German Shepherd	*M. bovis*	Culture & PCR	Gay *et al.* 2000
1	South Korea	1992	8	ME	Cross Breed	*M. tuberculosis*	PCR	Park *et al. 2016*
1	Czech Republic	1990's	NR	NR	NR	*M. bovis*	Culture	Pavlik *et al.* 2002
1	UK	2002	5	NR	Golden Retriever	*M. bovis*	Culture	Barber *et al.* 2004
2	UK	1992	NR	NR	NR	*M. bovis*	Culture	DeLisle, 1992
1	New Zealand	1963	7	ME	Fox Terrier	*M. tuberculosis*	Culture	Cordes *et al.*1963
1	New Zealand	1952	NR	NR	NR	*M. bovis*	Culture	Dodd, 1952
1	Hungary	2004	2	ME	West Highland White Terrier	*M. tuberculosis*	Culture	Pavlik *et al.*2005

1	Czech Republic	2004	5.5	FE	Doberman	*M. tuberculosis*	Culture	Pavlick *et al.* 2004
12	South Africa	2012	Mixed	Mixed	Mixed	*M. tuberculosis*	IGRA	Parsons *et al.* 2012
14	UK	1955	Mixed	Mixed	Mixed	*M. tuberculosis*	Culture	Hawthorne *et al.* 1957
137	UK, USA, Central Europe	Up to 1955	Mixed	Mixed	Mixed	*M. tuberculosis*	Culture	Snider *et al.* 1971a
44	UK, USA, Central Europe	Up to 1955	Mixed	Mixed	Mixed	*M. bovis*	Culture	Snider *et al.* 1971a
22	UK	1940	Mixed	Mixed	Mixed	*M. tuberculosis*	Culture	Lovell and White, 1940
1 1 1	3	2009	7 6 3	ME ME NR	Patterdale Terrier Border Terrier Cross Breed Terrier	*M. bovis*	Culture	Van der Burgt *et al.* 2009
20	Italy	1929	Mixed	Mixed	Mixed	*M. tuberculosis*	Culture	Paltrinieri, 1930
16	UK	1910's	Mixed	Mixed	Mixed	*M. tuberculosis*	Culture	Stableforth 1914
25	UK	1930's	Mixed	Mixed	Mixed	*M. tuberculosis*	Culture	Innis, 1940
13	Finland	1910-1935	Mixed	Mixed	Mixed	*M. tuberculosis*	Culture	Hjarre, 1939
1	USA	2004	9	FN	Golden Retriever	*M. tuberculosis*	Culture	Sykes *et al.* 2007
4	USA	1966-1968	Mixed	Mixed	Mixed	*M. bovis*	Culture	Snider, 1971b
8	USA	1962-1972	Mixed	Mixed	Mixed	*M. tuberculosis*	Culture	Liu, 1980
1	UK	1928	NR	NR	NR	*M. tuberculosis*	Culture	Moore, 1928
1	UK	1930	NR	NR	NR	*M. tuberculosis*	Culture	Thayer *et al.*1930

1	UK	1932	NR	NR	NR	*M. tuberculosis*	Culture	Adams and Vokald 1932
1	UK	1933	NR	NR	NR	*M. tuberculosis*	Culture	Steinback and Deskowitz 1933
494	Central Europe	Up to 1951	Mixed	Mixed	Mixed	MTBC	Culture	Cheyrolles 1951

10.3.1 Signalment

Within the cohort of 1012 dogs identified as being infected with MTBC organisms, 515 (50.9%) were definitely speciated. Only three organisms of the MTBC were identified; *M. tuberculosis* (n=297, 57.7%)*, M. bovis* (n=212, 41.2%) and *M. microti* (n=6, 1.2%).

The median age of dogs infected with *M. tuberculosis* was five years (range 0.5-9 years)*, M. bovis* was six years (range 1-9 years) and *M. microti* four years (range 4-7 years). Included in this study are a single group of 97 Foxhounds (9.6% of 1012 cases) affected during the same outbreak of *M. bovis* disease in a working kennel. The breed of the dog was only recorded in an additional 28 instances (2.8%) comprising; five cross-breeds, three Yorkshire terriers, three Golden Retrievers, two Doberman Pinchers, two German Shepherds, two Fox terriers, and one each of Patterdale terrier, Border terrier, West Highland White terrier, Basset hound, Beauceron, Podengo Iberico, Springer Spaniel, Beagle, Cavalier King Charles Spaniel, Labrador and Catalunyan sheepdog.

10.3.2 Clinical Signs

Of the 1012 cases identified in this study 156 (15.4%) were individually described with accompanying clinical detail; 96 dogs were infected with *M. tuberculosis,* 57 were infected with *M. bovis* and three were infected with *M. microti*. The clinical presentation of tuberculosis in these cases falls into four major categories based on the clinical presentation of the disease, which in order of frequency comprise; primary pulmonary, primary abdominal, disseminated or miscellaneous signs. Notably, pyrexia was an intermittently reported feature found amongst all presentations of this disease.

Primary pulmonary tuberculosis was described in 62 of 96 cases (64.6%) of *M. tuberculosis,* 34 of 57 (59.6%) cases of *M. bovis* and one of three (33.3%) of cases of *M. microti* infections, where the clinical presentation is detailed (Figure 10.1). Furthermore, it was frequently referred to as the most commonly noted form of disease in publications detailing groups of cases that did not reproduce individual case details.

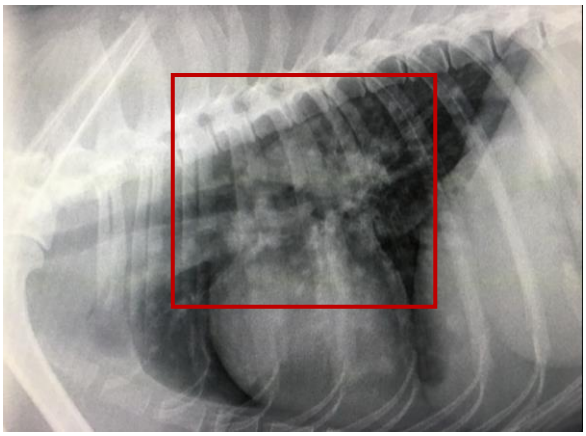

Figure 10.1: A latero-lateral radiograph of a Labrador infected with *M. bovis* displaying peri-bronchial pathology typical of primary pulmonary pathology. *Image courtesy of Scott Lawson MRCVS.*

The pulmonary manifestations of canine tuberculosis was shown to begin, as expected, with respiratory signs; typically a husky, non-productive cough persisting for a number of weeks. This then often progressed to a more severe cough which typically then became productive. Eventually dogs became dyspnoeic with a restrictive breathing pattern and at this point frequently developed pulmonary effusion and (occoasionally) a secondary pneumothorax. The lymph nodes in the thoracic cavity of these cases wre ubiquitously enlarged and it was usual for them to contain an internal area of necrosis when examined at *post mortem* examination (Figure 10.2).

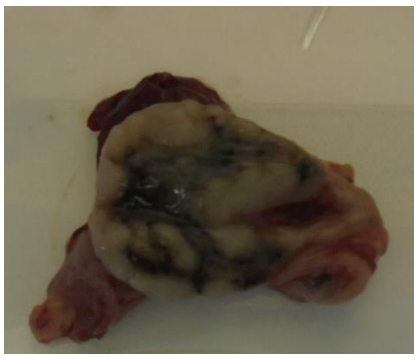

Figure 10.2: An incised bronchial lymph node removed *post-mortem* from a four-year-old male entire Foxhound dog infected with *M. bovis* demonstrating the lymphademomegaly and internal necrosis typical of canine pulmonary tuberculosis.

Cases presenting with primary abdominal tuberculosis represented approximately one third of cases. This form of disease was documented in 32 of 96 (33.3%) dogs with *M. tuberculosis* infection, 17 of the 57 *M. bovis* cases (29.8%) and one of the three (33.3%) *M. microti* cases described.

Lethargy, hyporexia/anorexia and the loss of body weight and condition were ubiquitous clinical signs reported among the dogs affected by primary abdominal tuberculosis (Figure 10.3). The weight loss recorded was frequently both rapid and marked; often reported as between 20% and 25% in just a small number of weeks. The liver was most frequently reported as the affected abdominal organ. The liver was often found to contain palpable abnormal (granulomatous) structures later in the course of disease in a very large proportion of cases. Other structures affected have been recorded to include; the pancreas, the stomach, the mesentery and the associated gastrointestinal lymph nodes. It has been noted that palpation of these abdominal granulomas often becomes easier to detect as the granuloma expands in size and the patient loses body condition. Associated with this, there may be intermittent vomiting and diarrhoea.

Figure 10.3: The loss of muscle condition from the temporal muscles (red box) of a three-year-old male neutered Ladrador dog infected with *M. bovis. Image courtesy of Sheena Lewis MRCVS.*

Disseminated disease was found in two *M. tuberculosis* cases (2.1%), four *M. bovis* cases (7.0%) and one *M. microti* case (33.3%). This form of the disease was often reported to have arisen insidiously and without any characteristic pattern to the clinical signs so that in all of these cases, these infections initially went undiagnosed. Eventually, all of these cases were euthanased due to the severity of disease which had developed. All seven cases presented at times with variable appetite, and protracted weight loss (though significantly slower and less severe than was seen with primary abdominal cases). At at least one assessment of routine haematology and serum biochemistry was abnormal in these cases; all cases displayed a pattern typical of a stress leukogram (mature neutrophilia, monocytosis, lymphopenia and eopsinopenia) though the neutrophilia was often described as marked or severe, non-regenerative anaemia and azotaemia. Notably, these abnormalities were not present every time that blood was evaluated and when the dogs were initially presented routine haematological and serum biochemical assessment was normally round to be unremarkable.

Four of these seven cases of disseminated tuberculosis (57.1%), including both of the *M. tuberculosis* infected dogs, one of the *M. bovis* infected dogs and the *M. microti* infected dog,

were diagnosed with dystrophic calcification, primarily affecting the kidneys of three dogs and primarily affected the liver in one case. All seven dogs were found to have elevated serum concentrations of ionised calcium, marked azotaemia and were presented to their primary veterinary surgeons for severe polyuria and polydipsia.

The remaining two *M. bovis* cases of the 57 with specific details available (3.5%) that did not fit into any of the three categories of tuberculous disease and are therefore classed as 'miscellaneous presentations'.

One case, an adult (exact age unknown) male neutered rescue Doberman presented with two focal ulcerated lesions, one on the flank (Figure 10.4) and one on the lateral digit of the right forelimb. Histological examination of the biopsied flank lesion revealed coalescing areas of granulomatous inflammation with central necrosis surrounded by large epithelioid macrophages and some neutrophils. Ziehl-Neelsen staining realed moderate numbers of acid-fast bacilli with mycobacterial morphology. Subsequent PCR testing of frozen biopsy tissue confirmed the presence of *M. bovis* DNA in the lesion. The dog was successfully treated with the surgical removal of the lesions, including amputation of the affected digit, followed by eight weeks of anti-tuberculous therapy (see below) and remains clinically well at the time of writing.

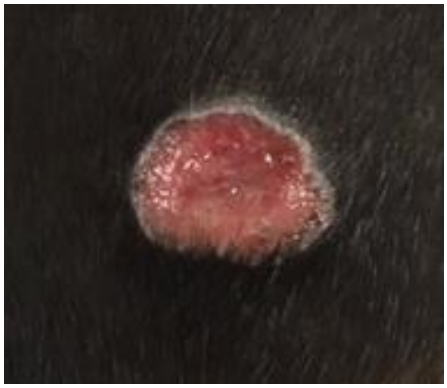

Figure 10.4: The ulcerated granulomatous lesion on the flank of a neutered male Doberman of unknown infected with *M. bovis. Image courtesy of Helen Dixon MRCVS.*

The final case in this series is a three-year-old male neutered Springer Spaniel with a three month history of chemosis, blepharospasm and epiphora following a superficial corneal injury of unknown cause. A superficial corneal lesion (Figure 10.5) developed over a number of weeks and slowly increased in size. It was non-responsive to therapy with fusidic acid eye

drops (Fuscithalmic™ [1% fusidic acid]; LEO Pharma, Denmark). Cytological examination of a fine needle aspirate of the lesion revealed granulomatous inflammation with non-staining "ghost" bacilli. Additional aspirates were found to contain acid-fast bacilli and one was PCR positive for *M. bovis* DNA. During this time the dog developed three dermal lesions (one on the lower lip and two on the cranial portion of the dorsum). Systemic treatment was instigated with anti-tuberculous therapy (see below) and topical therapy for the ocular lesion with moxifloxacin drops (Vigamox™; Alcon, UK). The lesions completely resolved within six weeks and the dog remains well at the time of writing.

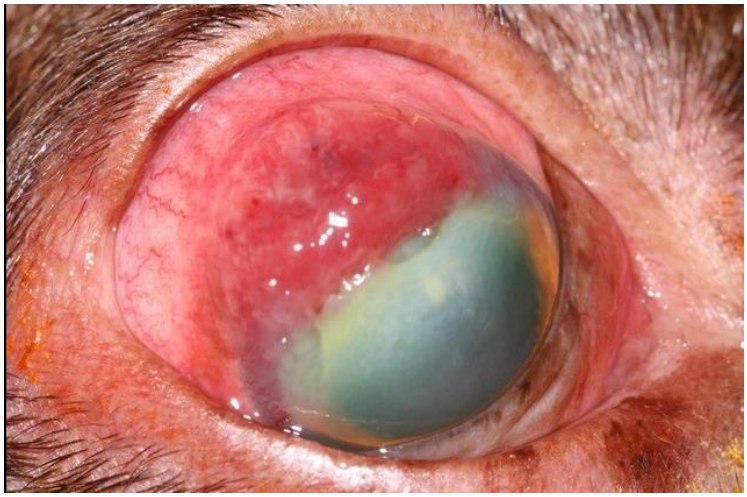

Figure 10.5: The corneal granuloma of a thee-year-old male neutered Springer Spaniel dog infected with *M. bovis*. *Image courtesy of Jess Florey MRCVS (Dick White Referrals).*

10.3.3 Diagnostic Tests

The diagnosis of mycobacterial infections, and MTBC infections in particular, are notoriously challenging to make as all of the available diagnostic tests have limitations of sensitivity and/or specificity.

For the majority of cases, evaluation of routine haematology and serum biochemistry were used as a first-line test when clinicians were presented with an unwell dog, however, the findings were typically unrewarding. The results, in the vast majority of cases for which they are available, were found to be within reference intervals. Occasionally, generally as the

severity of the disease advanced, non-specific changes occured, such as a stress leukogram (mature neutrophilia, monocytosis, lymphopenia and eopsinopenia). The mature neutrophilia seen has occasionally been described as marked or severe and in rare cases there is a band neutrophilia (a 'left-shift'). A number of tuberculous dogs have been found to have a non-regenerative anaemia; which may result from chronic inflammation. Organ specific changes, for example elevations in liver enzyme activity, were typically only found to occur when the relevant organ system was implicated in the disease process.

Diagnostic imaging (computed tomography, radiography and ultrasonography) were used in a number of cases, particularly those diagnosed in recent years. The findings were useful for establishing the extent of the pathology associated with infections and changes in diagnostic imaging was reported in a number of cases as an aid to monitoring response to therapy and as an indicator of when treatment could be discontinued.

Accessible lesions were often sampled by fine needle aspiration and cytological evaluation has additionally been conducted on bronchial alveolar lavage fluid (*N.B.* this procedure should only be undertaken in cases where tuberculosis is suspected after careful consideration and with appropriate protective equipment). Cytological findings from these cases represent the typical mammalian inflammatory response to mycobacteria; active macrophages dominate, variable numbers of neutrophils and lymphocytes and fewer plasma cells. Routinely used Romanovsky-type stains (e.g. DiffQuick™; Richard Allen Scientific, UK) may highlight intra- and extracellular mycobacteria as non-staining, so called "ghost" bacilli as their lipid walls precent the uptake of stain. Acid-alcohol protocols (e.g. Ziehl-Neelsen) are needed to actively stain mycobacteria.

A similar inflammatory infiltrate to cytological findings was ubiquitously reported in histological evaluations of lesioned tissue sections taken from tuberculous dogs but in addition the granulomatous structure was also visible. Canine granulomas of various structure have been described; the most commonly identified were well structured granulomas comprising large, often circular or ovoid, encapsulated areas of densely packed epithelioid macrophages, Langerhan's cells and multinucleated giant cells. The peripheral macrophages were frequently

reported as admixed with lymphocytes that were occasionally seen to form small aggregates. In the central regions, irregular areas of necrosis containing cell debris, intact and degenerate neutrophils were variably recorded. The areas of central necrosis were notably missing from all three cases of *M. microti* infection where the histological assessment of tissue was available. In a small number of cases, the (pyo)granulomatous inflammation was found to occur outwith a typical granulomatous structure but instead macrophages and neutrophils simply infiltrated the affected organs in large numbers and only formed small aggregates. The histological findings in cases that were highly suggestive of mycobacterial infections were examined by specialist stains to look for aetiological agents. The Ziehl-Neelsen protocol identified variable numbers of acid-fast bacilli in the sections were this was reported, in line with findings reported for mycobacterial infections in other species (Brougham *et al.* 2013. In many of the cases reviewed in this study, the detailed findings of acid-fast staining were frequently omitted from case reports (*i.e.* they were often simply referred to as either positive or negative) and so a more detailed analysis was not possible.

A number of the examined reports noted that these granulomatous structures and/or histological findings may also arise in the absence of an infectious agent but due to foreign body, canine histiocytic disorders, canine sterile granuloma syndrome or neoplasia (e.g. histiocytoma).

In the UK and the USA, the only diagnostic test which can lead to official confirmation of a tuberculous infection in a dog is specialist mycobacterial culture conducted by the Animal and Plant Health Agency, Weybridge or the United States Department of Agriculture, Animal and Plant Health Inspection Service respectively (USDA, 2017; Middlemiss and Clark, 2018). Despite this, no sensitivity data are publically available for this technique with respect to canine submissions. Logically, culture tests are assumed to have perfect (100%) specificity for all submissions processed at these laboratories.

In human medicine, tuberculosis is increasingly being diagnosed with the use of molecular assays; either PCR or quantitative PCR (qPCR). This has, in large part, been driven to decrease the time taken to make a diagnosis compared to culture which can take 2-4 weeks

for *M. tuberculosis,* 6-8 weeks for *M. bovis* and up to 12-16 weeks for *M. microti* (Reuter *et al.* 2006; Emmanuel *et al.* 2007). In the UK, Mycobacterial Reference Laboratories, such as the one at Leeds University Teaching Hospital, are offering veterinary clinicians the most robust PCR assays for mycobacterial infections. This laboratory utilises the GenoType MTBC assay (Hain LifeScience, Germany) which allows the discrimination between all members of the complex in one rapid (five hours) assay (Hain LifeScience, 2019). However, this assay is validated for the assessment of liquid starting material (typically liquid cultures or expectorated sputum samples) where most veterinary submissions are cytological aspirates or tissue biopsies and this may have an, as yet, effect on the test sensitivity. In addition, veterinary submissions may also be formalin-fixed tissues being tested following routine histological examination and whilst this process is known to fragment extracted DNA, the effect that this may have on test sensitivity is as yet unknown. Despite this, positive results have been obtained from formalin-fixed canine tissue, even without visible acid-fast bacilli (Sharp *et al.* 2019).

Parsons *et al.* (2012) were the first to use a commercial interferon gamma release assay (IGRA) to test dogs for *M. tuberculosis* infections. This test, the QuantiFERON™ TB Gold assay is used to diagnose latent tuberculosis infections (LTBI) in people (Tilahun *et al.* 2019). The IGRA assays incubate whole blood or isolated cells (peripheral blood mononuclear cells [PBMC]) with mycobacterial antigens; if patients are infected with mycobacteria that posess those same antigens then antigen-specific T-cells will recognise those antigens, produce and secrete interferon-gamma (Wood and Jones, 2001). This can then be quantified by ELISA and compared to unstimulated samples from the same patient; a significant difference indicates to infection (Wood and Jones, 2001). In humans, the QuantiFERON™ TB Gold assay is reported to have a sensitivity of 82.3% (95% confidence interval; 77.7% to 85.9%) and specificity of 82.6% (95% confidence interval; 78.6% to 86.1%) for LTBI (Takwoingi *et al.* 2019). The study by Parsons *et al.* (2012) screened high risk dogs (resident with humans that had active *M. tuberculosis* infections) and showed that a number generated positive results to the QuantiFERON™ TB Gold assay.

The QuantiFERON™ TB Gold test contains three combined MTBC antigens; 6kDa early-secreted antigenic target (ESAT-6), 10kDa culture filtrate protein (CFP-10) and Tb7.7 (Bernitz *et al.* 2019). Only the latter of these is specific to *M. tuberculosis,* the former two are both common to MTBC organisms which encode the RD-1 region of the MTBC genome (*i.e.* all MTBC organisms excluding *M. bovis*-BCG and *M. microti*) (Leem *et al.* 2018; Bernitz *et al.* 2019). Therfore a positive response of a dog to these antigens likely indicates infection with a RD-1-positive MTBC mycobacteria rather than just *M. tuberculosis* as implied by Parson *et al.* (2012), for example buffalo infected with *M. bovis* have been shown to give positive results to the same test (Bernitz *et al.* 2019). No further studies have expanded on the use of the QuantiFERON™ TB Gold test for dogs and, to the author's knowledge, it is not currently available to veterinary clinicians.

Another IGRA, based on the protocol used for the feline assay, was developed during an outbreak of *M. bovis* in a kennel of Foxhounds (Chapter 5; O'Halloran *et al.* 2018). This assay uses three separated antigen pools; purified protein derived (PPD) from *M. bovis* (PPDB), PPD from *M. avium* (PPDA) as well as a combination of ESAT-6 and CFP-10. This separation of antigens theoretically allows for the discrimination between canine infections with *M. tuberculosis* and *M. bovis* (both of which would give rise to the same pattern of response) and *M. mircoti.* However, no dogs infected with *M. microti* have been tested with this assay to date. Additionally, as with cats (Rhodes *et al.* 2008), a number of the Foxhounds tested that were infected with *M. bovis* did not give a significant interferon gamma response to the ESAT-6 and CFP-10, as would be expected from animals infected with *M. microti.* Therefore, whilst this test would appear helpful in diagnosing MTBC infections in dogs, it may not always discriminate between the different species. This IGRA test is now available to clinicians at Biobest Laboratories, Edinburgh.

Serological assays are for the identification of tuberculosis infections in humans and other animal species (Waters *et al.* 2017; Bezos *et al.* 2018; Chen *et al.* 2018; Infantes-Lorenzo *et al.* 2019). The advantages of these assays is that they are comparatively inexpensive and rapid in relation to other tests (Waters *et al.* 2017; Bezos *et al.* 2018). In the context of cattle

and wildlife species, the acquisition of the small volume of serum required to run the assay is practically easier than conducting tests such as the tuberculin skin test which requires the same individual to be handled twice, usually 72 hours apart (Infantes-Lorenzo *et al.* 2019). Of the available commercial assays, the Chembio Dual Path Platform VetTB Assay for Cervids has been used in dogs but using a test cut-off point that maintains a high specificify, the sensitivity was found to be poor (O'Halloran *et al.* 2018). Therefore it is likely that, for the forseeable future, this and similar serological tests currently only used in research, will only be useful for screening large groups of at-risk animals rather than being a reliable tests for individual dogs.

10.3.4 Treatment and Outcome

The treatment of canine tuberculosis is contentious as all of the MTBC organisms isolated from the cases considered in this review can cause human disease and the inappropriate treatment of pets may inadvertently lead to drug resistant isolates, further risking public health. Indeed, the treatment of *M. tuberculosis* infected pets has been strongly discouraged on public health grounds and owners of such animals should be referred to the appropriate public or general health professionals (O'Halloran and Gunn-Moore, 2017).

By contrast, *M. microti* has only ever been identified in six dogs and although a small number of people have developed disease due to this organism, no cases have been linked to transmission from a dog (Emmanuel, 2007).

The public health risk from infections with *M. bovis* may be considered to sit somewhere between that of *M. bovis* and *M. tuberculosis.* Unlike *M. tuberculosis* which represents the major cause of human disease, *M. bovis* now causes only approximately 1% of human tuberculosis cases in the UK (Glaziou *et al.* 2018). However, zoonotic cases due to dogs have been confirmed or strongly suspected in recent years (Ellis *et al.* 2006; O'Halloran *et al.* 2018). Despite this, Public Health England deem the risk of zoonotic tuberculosis due to pets to be "very low" (Public Health England, 2014).

Treatment details for tuberculosis was reported in 22 cases recorded cases in this study; including the 19 cases were management was supervised by the author and nine previously published reports. In 20 cases the dogs were infected with *M. bovis* and one dog was infected with *M. microti.* All except one *M. tuberculosis* infected dog euthanased at the time the diagnosis was confirmed.

The treatment protocol for the 19 dogs supervised by the author was based on a triple antimicrobial combination of drugs, adapted to dogs from the published guidance for cats (O'Halloran and Gunn-Moore, 2017). This outlines combination therapy comprising rifampicin, a macrolide and a quinolone administered daily. This class combination has been chosen for several reasons. Firstly, streptomycin should be kept as a last-resort antibiotic for human patients and should not be used in dogs (Engelmann *et al.* 2014). Secondly, isoniazid can cause severe adverse neurological effects that has led to euthanasia of a dog (Sykes *et al.* 2007). Thirdly, pyrazinamide is useful ineffective for the treatment of *M. bovis* infections (de Jong *et al.* 2005). And quinolones, which have documented anti-mycobacterial activity are well tolerated by dogs (Govendir *et al.* 2011).

All 19 dogs received rifampicin (generic), an essential component of anti-tuberculous therapy, at a dose rate of 10-15mg/kg by mouth (PO) every (q) 24 hours (Munir *et al.* 2019). Three dogs (15.8%) subsequently developed pruritic skin eruptions (Figure 10.6), putatively due to the rifampicin as a similar reaction has been reported for cats (O'Halloran and Gunn-Moore, 2017), though these cases appeared more severe than has been documented in cats, and the lesions healed subsequent to the withdrawal of the drug. In these cases, rifampicin was replaced with doxycycline (Ronaxan™; Boehringer Ingelheim, Germany) at a rate of 10mg/kg PO q 24 hours for the remaining duration of therapy.

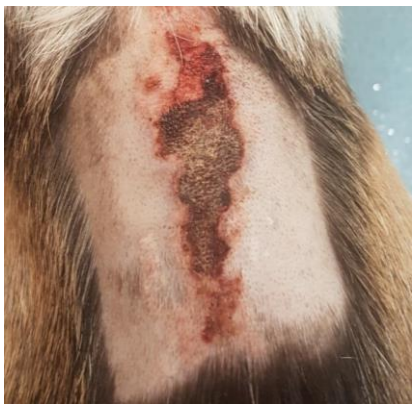

Figure 10.6: A skin eruption on the back of a 6-year-old neutered male Bealge dog with an *M. microti* infection five weeks after starting therapy with rifampicin at 11.5mg/kg po q24 hours. The lesion extends craniodorsally (top to bottom of the picture) approximately 5cm by 2cm laterally. *Image courtesy of Alex McInroy MRCVS.*

One dog receiving rifampicin became inappetant and evaluation of serum biochemistry revealed a serum alkaline phosphatase (ALP) to ten-times the upper reference interval. Rifampicin therapy was discontinued immediately, doxycycline was started at previously stated doses and the dog's appetite returned within 24 hours. Serum biochemistry was not repeated but the dog made a full clinical recovery.

All 19 dogs either received a macrolide alongside the rifampicin; ten dogs (52.6%) were treated with the drug azithromycin (Zithromax™; Pfizer, UK) at a dose rate of 10-15mg/kg po q24 hours and the remaining nine dogs (47.4%) were treated with clarithromycin (generic) at a dose rate of 7.5-15mg/kg po q12 hours. Azithromycin was chosen for smaller sized dogs (~15kg or less) as this drug is available as a reconsitited suspension allowing the amount administered to be accurately measured. By comparison, clarithromycin is available as 250mg or 500mg tablets (NHS, 2019), and the former size is an optimal size for a ~16kg dog.

In cases with respiratory tuberculosis, azithromycin has been argued for as the optimal macrolide drug choice. This drug characteristically concentrates in lung tissue at 100-fold greater levels than in the circulation (Hand *et al.* 2001). A further interesting characteristic is the ability of azithromycin to concentrate within inflammatory cells, particularly neutrophils and monocytes within the bloodstream, and tissue macrophages (Mandell *et al.* 2001). The ongoing inflammatory process in the lung of these dogs may recruit azithromycin-laden

neutrophils from the circulation which might then unload the drug in the presence of mycobacteria within inflammatory sites (Hand *et al.* 2001). Inflammatory cells can therefore act as “Trojan horses” delivering local concentrations of azithromycin that are several orders of magnitude greater than that in plasma (Blumer, 2005).

The final drug in the triple combination is a quinolone. Pradofloxacin is recommended in cats due to its increased safety profile in this species (Sykes and Blondeau, 2014). Pradofloxacin has proven efficacy against mycobacteria and also has an excellent safety profile in dogs and so was used to treat seven of these 19 cases (Govendir *et al.* 2011; Boothe *et al.* 2018). The dose rates ranged from the UK licensed dose of 3-5mg/kg PO q 24hrs up to include the USA licensed dose of 7.5mg/kg PO q24hrs (Anon, 2018; Boothe *et al.* 2018). One dog, a four-year-old male neutered Greyhound developed neutropenia after being on the higher dose of pradofloxacin and was transitioned onto marbofloxacin (Marbocare P™; Animalcare, UK) at a dose of 2mg/kg PO q24 hours and the neutropenia resolved. Five of the dogs had been given empricial marbofloxacin therapy (at the previously stated rate) before the diagnosis of tuberculosis was made and so this was continued. Six dogs were treated with enrofloxacin (Baytril™; Bayer, Germany) at 5-10mg/kg PO q24hrs. One of the treated dogs was a one-year-old Staffordhire Bull terrier and there were concerns about using quinolones in a dog of this age due to the potential for damage to developing cartilage, especially given the anticipated duration of therapy (Sebastian, 2007). For this dog, doxycycline was subsitituted for the quinolone component of the triple antibiosis (Ronaxan™; Boehringer Ingelheim, Germany) at a rate of 10mg/kg PO q 24 hours.

Three dogs with pulmonary disease also presented with productive coughs. Due to concerns that this might pose a zoonotic risk to their owners, anti-tussive therapy was instigated. The dogs received 0.5-1mg/kg codeine phosphate PO q6-8 hours.

These 19 cases were treated for a minimum of 12 weeks and for at least eight weeks following complete resolution of clinical signs. Where internal pathology had been noted then the treatment was extended until this had either resolved or had remained unchanged for at least six weeks *i.e.* any changes were no longer actively improving or deteriorating. Treatment

duration ranged from 14 weeks to 38 weeks (median 20 weeks). All dogs responded favourably to therapy and to the authors' knowledge are still alive at the time of writing.

The treatment protocols for three dogs are presented in the literature. One dog with abdominal tuberculosis was treated with rifampicin (generic) at 10 mg/kg administered PO q24h, clarithromycin (generic) at 12 mg/kg PO q12h, and enrofloxacin (Baytril™; Bayer, Germany) 5 mg/kg administered PO q12h for three months when the enrofloxacin was discontinued and the remaining two former drugs were continued for a further three months (Englemann *et al.* 2014). The dog fully responded to therapy and the authors report it was well 31 months after the initial diagnosis (Englemann *et al.* 2014).

The second dog also presented with abdominal tuberculosis (due to *M. bovis* infection) but was only treated with enrofloxacin monotherapy (dose rate unknown) (Shrikrishana *et al.* 2009). The dog only partially responded to therapy and was euthanased shortly after therapy was instigated.

The final dog was resident in Brazil and it was initially based on the presumptive diagnosis of Ehrlichiosis and so was treated with chloramphenicol (50 mg/kg PO q12 hours), dexamethasone (0.30 mg/kg PO q24 hours), and fluids (Rocha *et al.* 2017). However, the treatment was ineffective and the dog progressed to death; *M. bovis* was confirmed at *post mortem* examination.

10.4 Discussion

This study, the largest review of canine tuberculosis, evaluated 1012 cases and markedly improves the current knowledge of this disease.

Infecting Mycobacteria

Historical reviews have suggested a 3:1 ratio of infections caused by *M. tuberculosis* and *M. bovis* (Birn *et al.* 1965; Clerx *et al.* 1992; Liu, 1980). However in this study found a decrease in this ratio to just 2:1. If only cases diagnosed in pet dogs (*i.e.* excluding the single large

outbreak of *M. bovis* in working Foxhounds in the UK) within the last ten years (2009-2019) are examined, then there is a clear inversion of this trend; there are twice the number of *M. bovis* cases compared to *M. tuberculosis.* Additionally, all six *M. microti* cases were diagnosed in this same ten year period. This likely reflects the continuing decline in human *M. tuberculosis* cases in the countries/regions from which these cases originated (mostly Europe, North America and New Zealand). The most common risk factor for a dog to present with an active *M. tuberculosis* infection in this study was still an exposure to a human with active tuberculous disease. The increase in *M. bovis* and *M. microti* cases may also reflect the increasing prevalence of these infections in countries such as the UK and/or increased diagnostic capacity (e.g. PCR) to separate out infections within the MTBC.

Signalment

This analysis found that young to middle aged adult dogs formed the majority of those infected with MTBC organisms with a range from six months to nine years. There was no significant difference between the age distributions of the dogs infected with *M. tuberculosis, M. bovis* or *M. microti.* There was no sex predeliction for infection in the group of cases where this information was available, though this was only a minority of cases. This distribution, with a complete absence of older and geriatric dogs, may suggest that dogs may develop a degree of immunity as they age, potentially through exposure to environmental mycobacteria.

Although the breed was only recorded in a very small group of cases (<3%), it is notable that most of the pedigree breeds reported were hound or terrier types. This may indicate that these hound and terrier type breeds have an increased susceptibility to MTBC infection, or it may reflect an increase in exposure e.g. to wildlife reservoirs due to the natural behaviours of these breeds such as scavenging and/or hunting small mammals. However, the very small number of cases where breed information is available makes it difficult to draw firm conclusions.

Interestingly, there was no representation of breeds previously reported as susceptible non-tuberculous mycobacterial infections such as Miniature Schnauzer's or Boxers (Malik *et al.* 1998; Sharp *et al.* 2019). Identification of the underlying genetics of these susceptibilities

would allow a greater understanding of the pathogenesis of mycobacterial infections and could lead to improvements in diagnosis and the development of new treatments.

Clinical Signs

For the first time, the clinical presentations of canine tuberculosis were grouped into four different clinical presentations which could be helpful as clinicians can detail the zoonotic implications e.g. pulmonary cases, which were found to be the most common are likely to pose the greatest risk of zoonotic transmission. The classification can also help with the prognosis e.g. all cases presenting with disseminated disease were fatal. The miscellaenous presentations reported here are critically important as this form of disease is almost certainly the most likely to be missed by clinicians who may not be suspicious of cases that do not present with the more commonly expected pulmonary form. Delays in making a diagnosis can increase the risk to owners by extending the period of exposure to an infectious dog but also might mean that the opportunity to successfully intervene is lost.

Diagnostics

Specialist mycobacterial culture remains the gold standard diagnostic test but it not always practically possible and action (such as the instigation of therapy or euthanasia of the dog) may need to be taken prior to the availability of the results. Further work to validate PCR and IGRA methods would be helpful so that all stakeholders (owners, clinicians, public health officials and veterinary health officials) can have a clearer understanding of the test performance under normal field conditions and so interpret results appropriately. Although serological assays remain an attractive option for future development, it appears likely that they will remain too insensitive for use in individual patients.

Treatment

The very small percentage of cases (2.0%) were treatment was attempted implies that canine tuberculosis may be seen as carrying a poor prognosis but this analysis found that it is in reality a readily treatable infection; a good outcome (*i.e.* long term remisison of clinical disease)

was achieved in 86% of cases and 100% of cases were triple combination therapy was utilised. However, treatment was often of long duration and onwers must be aware of this before therapy is instigated. Also of significance, all cases of disseminated disease were euthanased on welfare grounds due to the severity of clinical signs and treatment would likely be unsuccessful in these dogs.

Although there is a report of the successful treatment of a case of *M. tuberculosis* infection with triple combination antimicrobial therapy, this should be discoraged based on the risk to public health from these dogs.

Side effects were reported with rifampicin therapy in a minority of cases which were severe including skin eruptions and both inappetance and induction of ALP activity. One dog was also reported as neutropenic, possibly due to chronic high dose pradofloxacin administration, but as this was a Greyhound, this may simply have been an incidental finding (Zaldívar-López *et al.* 2011). Dogs receiving anti-tuberculous therapy require careful monitoring and some clinicians may consider repeating haematological and serumbiochemical evaluation at regular interval (e.g. monthly) or if the dogs appear to become unwell whilst on treatment.

Conclusion

This analysis over over 1000 canine tuberculosis cases found that all cases were caused by infections with one of three MTBC organisms, *M. tuberculosis, M. bovis* or *M. mircroti.* This study found that in recent years the pattern of infections has changed with a relative decline in the significance of *M. tuberculosis* as compared with *M. bovis* and the first documented canine infections with *M. microti.* All cases conformed to one of four clinical groups based on the presenting signs; primary pulmonary, primary abdominal, disseminated or miscellaneous. For cases other than dogs with disseminated disease, treatment with triple combination antimicrobial therapy lead to good clinical outcomes but the treatment of dogs with *M. tuberculosis* remains against advice due to the public health risk.

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Chapter 11: Investigating the Early Response of Canine Bone Marrow Derived Macrophages to Mycobacteria.

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Abstract

Murine bone marrow derived macrophages (BMDM) and human monocyte derived macrophages grown in colony stimulating factor 1 (CSF-1) have been widely used to study macrophage responses to infectious agents as well as Toll-like receptor (TLR) agonists. It was hypothesised that recombinant CSF-1 could be used to reliably generate canine BMDM in order to characterise them, examine the effect of TLR agonism and investigate the early (initial 24 hours) response of these cells to mycobacterial infection. The culture of canine bone marrow cells for 10 days in recombinant human, porcine or canine CSF-1 at appropriate concentrations produced a pure population of BMDM that were adherent, morphologically resembled macrophages and expressed surface proteins typical of macrophages (as described in other species: the CSF-1 receptor, CSF-1, ADGRE-1, CD172a, CD163, CD16 and CD14). Canine BMDM could be generated from bone marrow cells that had been cryopreserved so that multiple experiments could be conducted on cells from a relatively small number of animals. The canine BMDM produced were phagocytic and therefore permissive to mycobacterial infection whereupon they produced the pro-inflammatory cytokines TNF-α and IL-6 but not nitrite. The BMDM were also found to display mycobactericidal activity during the early phase of infection. Gene expression and cytokine production of canine BMDM in

response to the TLR4 agonist lipopolysaccharide, attenuated *M. bovis* bacille Calmette-Guérin and virulent *M. bovis* showed significant differences to the canine macrophage-like DH82 cell line indicating that the latter is not a good resource for the study of canine mycobacterial infections.

11.1 Introduction

Cells of the monocyte-macrophage lineage (MML) and their response to mycobacterial infection is considered in Section 1.6. These MMLcells include bone marrow progenitor cells, circulating cells of the MML and tissue resident macrophages. The process of differentiation and maturation of macrophages from their progenitors is driven and regulated by the haemopoietic growth factors colony stimulating factor 1 (CSF-1) and interleukin (IL)-34 which share a common receptor (Hume, 2008; Fairbairn *et al.* 2011).

Cells of the MML are frequently the first cells of the innate immune system to interact with any potential pathogen and their early response can have profound effects on the outcome of infection including pathogen killing and clearance, or persistence and the development of clinical disease (Burkey *et al.* 2009; Szatmary, 2012; Pahari *et al.* 2018). Macrophages represent the immunological niche for the persistence of mycobacteria, to this end they have evolved a number of mechanisms for the subversion of mycobacterial killing; some of which are considered in Section 1.6.5, making tem one of the most critical central cells to the host-pathogen interaction and pivotal in the aetiopathogenesis of mycobacterial infections.

Microbial pattern-recognition receptors (PRRs) are an essential early component of innate immunity, they detect and recognise conserved pathogen-associated molecular patterns (PAMPS), resulting in the activation of stimulated MML cells (and neutrophils) as part of the host response to eradicate invading pathogens whilst maintaining immunological tolerance by recognition of self (Burkey *et al.* 2009; Szatmary, 2012; Pahari *et al.* 2018). An important class of mammalian PRRs is the Toll-like receptor (TLR) family which recognise a wide range of microbial pathogens and pathogen-related products (Schneberger *et al.* 2011).

A number of studies which investigated the response of macrophages to mycobacteria in humans and mice demonstrated that stimulation of TLR2 and TLR4 receptors, as well as TLR1 and TLR6 by mycobacteria-derived ligands results in downstream activation of the nuclear transcription factor NF-κβ which in turn leads to the up-regulation of genes encoding pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 (Underhill *et al.* 1999; Quesniaux *et al.* 2004; Bulut *et al.* 2005).

While methods exist for the isolation and/or culture of MML cells from bone marrow across several species, this requires *post mortem* material which is not always readily accessible (Kapetenovic *et al.* 2012). Therefore, utilisation of a cell line that accurately mimics the response of *ex vivo* MML would be beneficial. The DH82 cell line was established from the neoplastic progenitor cells of canine malignant histiocytosis and was characterised as histiocytic in origin based on light microscopic and ultrastructural morphology (Wellman *et al.* 1988). Since then it has been used as a model to investigate the canine response to intracellular pathogens including *Leishmania spp.,* canine distemper virus and extracellular pathogens such as *Mycoplasma canis* (Puff *et al.* 2009; Michaels *et al.* 2016; Soto *et al.* 2019)

The aim of this study was to utilise recombinant CSF-1 to generate canine macrophages from bone marrow (BMDM) in order to characterise them, examine the effect of TLR agonism and investigate the early (initial 24 hours) response of these cells to mycobacterial infection whilst comparing them to the established DH82 cell line in order to evaluate the usefulness of both systems as models for studying canine mycobacterial infections.

11.2 Materials and Methods

11.2.1 Isolation of Bone Marrow Cells

Bone marrow was acquired from dogs and cats that were euthanased at the Royal (Dick) School of Veterinary Studies for reasons unrelated to this project. All animals came from the Edinburgh Dog and Cat Home and were euthanased because of a history of human-directed aggression or severe animal-directed aggression which precluded the rehoming of these animals to members of the public. No animals showed clinical signs of infectious disease, including mycobacterial infection, on veterinary examination prior to euthanasia.

All euthanasias were performed by intravenous injection of pentobarbitone at a dose of ≥1mg/kg administered by the author (a UK qualified and registered veterinary surgeon), following prior administration of oral sedation (acepromazine, ACP, 0.05mg/kg [ACP; Elanco, UK], phenobarbitone 3mg/kg [Epiphen, Vetoquinol, UK] and diazepam 1mg/kg [generic]) by the attending handlers, or by intramuscular injection of butorphanol 0.2mg/kg [Torbugesic; Zoetis, USA], 0.05mg/kg ACP and 0.05mg/kg medetomidine [Sedator; Dechra, UK] administered by the author.

The sedation and euthanasia procedures as well as the use of cadavers for research purposes had prospective ethical approval from the University of Edinburgh Veterinary Ethics Research Committee and was adhered to throughout.

The long bones; femur, humerus and ribs, were removed *post-mortem* from euthanased animals within one hour of euthanasia; the fascia and connective tissues were removed with a size 22 scalpel blade and the bones were then stored in PBS on ice until further processing (within four hours of euthanasia). The bones were surface decontaminated by immersion in 70% ethanol for five minutes which was then removed by rinsing the bones in sterile PBS. A hand saw with a sterile blade attached was used to trisect the bones by incising through the diaphysis twice in order to expose the medullary cavity.

Bone marrow cells were removed by flushing the central cavity with warmed (37°C) RPMI 1640 media supplemented with anticoagulant; sterilised sodium citrate, added to a final concentration of 3.2% by volume, through a 20mL syringe and 22 gauge needle. The flushed, cell-containing media was collected into 50mL sterile plastic tubes which were centrifuged at 600 x *g* for five minutes before the supernatant was removed and discarded. The cell pellet in each tube was re-suspended in approximately 10mL of an ammonium chloride erythrocyte lysis buffer (composed of 8.26 g ammonium chloride, 1.0 g potassium bicarbonate, and 0.037 g EDTA in 1dL of water), the suspension was transferred to a clean tube and incubated in the dark for seven minutes before the tube was filled with RPMI 1640 media supplemented with 20% foetal bovine serum. The suspension was passed through a 40µm Corning cell strainer (Corning Life Sciences, Germany) to remove any remaining debris and the flow through was again centrifuged at 600 x *g* for five minutes before the supernatant was removed.

The cell pellets were re-suspended in a single mL of non-supplemented RPMI 1640 media and all suspensions were combined into a single clean tube at which time 10µL was removed and mixed with an equal volume of 2% Trypan blue stain prior to counting the number of live cells using a haemocytometer.

11.2.2 Cryopreservation and Recovery of Bone Marrow Cells

Cells were centrifuged at 600 x *g* for five minutes and the supernatant was discarded. Cold (4^{o}C) cell freezing media comprised of heat inactivated FBS (Gibco, UK) containing 20% dimethyl sulfoxide (DMSO; Sigma Aldrich, UK) by volume was added dropwise to the cell pellet with manual agitation of the tube throughout. The volume added was sufficient to give a final cell concentration of $5x10^{7}$ cells/mL. This cell suspension was divided into 1mL cryovials (Thermo Fisher Scientific, USA) which were placed into a pre-chilled (-20^{o}C) Mr Frosty^{TM} cell cooler containing isopropanol.

The Mr Frosty^{TM} cell coolers containing the cell vials were placed at -80^{o}C for 72 hours after which time the cell vials were removed and transferred to a liquid nitrogen tank for long term storage. When needed, each vial was defrosted in a water bath pre-warmed to 37^{o}C. The

thawed cells were transferred to a 15mL Falcon and 10mL of warmed complete culture media was added dropwise with agitation. The cells were centrifuged at 600 x *g* for five minutes and the supernatant was removed. Cells were re-suspended in 10mL of complete culture media (RPMI 1640 containing 100µg/ml L-glutamine, 10% foetal bovine serum, 100µg/ml penicillin, 100U/ml streptomycin, $5x10^{-5}$M 2-mercaptoethanol and non-essential amino acids). Cell viability counts were performed using Trypan blue staining (Section 11.2.1).

11.2.3 Culture of Bone Marrow Derived Macrophages

When recovered from cryopreservation or removed from long bones, viable bone marrow cells (as determined by Trypan blue staining; Section 11.2.1) were diluted to a density of $1x10^6$/mL in complete culture media. The cell suspension was plated into appropriately sized Sterilin™ treated tissue culture plastic dishes (Thermo Fisher Scientific, UK). The growth factor recombinant colony stimulating factor 1 (r-CSF-1) was added at an appropriate concentration (see Section 11. 3). An equal volume of fresh complete culture media was added to cells after three days in culture at 37°C/5% CO_2 along with sufficient r-CSF-1 so as to reach the same final concentration as on Day 0. After six days in culture, all media was removed, discarded and replaced with the same volume and quantity of r-CSF-1 as at time Day 0.

11.2.4 Determination of Cell-surface Molecule Expression by Single and Dual Colour Flow Cytometry

At four time points during macrophage differentiation; Day 0, 3, 6 and 10 the media was removed from the bone marrow cell cultures and transferred into 50mL Falcon tubes. The adherent cell layer was washed twice with sterile PBS to remove serum remnants. Following this, 10mL of non-enzymatic cell dissociation solution (Sigma Aldrich, UK) was added to the flask and incubated at 37°C until the cells visibly detached from the plastic. The cell solution was also transferred to a 50mL Falcon tube. All tubes were centrifuged at 600 x *g* for five minutes at room temperature, the supernatant was discarded and the cell pellets were each re-suspended in 1mL of complete cell culture media and combined into a single tube. Cell viability counts were performed using Trypan blue staining (Section 11.2.1). Cells were diluted

to a final concentration of $5x10^6$/mL in complete culture media and 200µL (*i.e.* $1x10^6$ cells) was added to the required number of wells of a round-bottom CELLSTAR® 96W Microplate (Sigma Aldrich, UK). The plate was centrifuged at 600 x *g* for five minutes at room temperature and the supernatants were discarded. Cells were washed twice in 100µL of sterile PBS and re-suspended in block buffer (PBS containing 2% heat inactivated normal goat serum [Abcam, UK]) for half an hour at room temperature. Cells were washed twice in 100µL of sterile PBS to remove any serum remnants. The fixable cell viability stain Zombie Violet™ (Zombie Violet™ Fixable Viability Kit; Biolegend, USA) was added to all wells, other than the unstained control well, at a dilution of 1:1000 (as recommended by the manufacturer) in 50µL per well of sterile PBS without serum. Unstained control cells were re-suspended in 50µL of sterile PBS. The plate was incubated in the dark for 20 minutes at room temperature.

Cells were washed twice in blocking buffer before primary antibodies including relevant isotype controls (Table 11.1) were added to the appropriate wells, diluted to the required concentration in 50µL of sterile PBS without serum. The plate was incubated in the dark for 30 minutes at room temperature. Cells were washed twice in blocking buffer before isotype- and species-matched secondary antibody was added to the appropriate wells (Table 11.1), including a well with just secondary antibody added with no primary antibody, diluted in 50µL of sterile PBS without serum. The remaining control cells were re-suspended in 50µL of sterile PBS only. The plate was incubated in the dark for 30 minutes at room temperature and cells were then washed twice in sterile PBS and re-suspended in 100µL of 2% paraformaldehyde solution and left for 10 minutes at room temperature. Cells were washed twice in sterile PBS and re-suspended in 100µL of flow cytometer buffer (sterile PBS containing 2% BSA, 2nM EDTA and 2nM sodium azide). The plate was sealed with an adhesive plate sealant to prevent evaporation and kept refrigerated at 4ºC overnight (approximately 16 hours) wrapped in foil to prevent exposure to light.

Table 11.1 Antibodies used for single and dual-colour flow cytometry

Surface molecule	Clone	Species specificity	Isotype	Conjugated flurochrome	Concentration	Supplier
CD14	TUK4	Mouse anti-human Mab	IgG2a	AlexaFluor647	10µg/mL	Abcam
CD172a	DH59B	Mouse monoclonal anti-bovine	IgG1	None	2.5µg/mL	Kingfisher
CD16	LNK16	Mouse monoclonal anti-human	IgG1	None	10µg/mL	Invitrogen
CD163	EDHu-1	Mouse monoclonal anti-human	IgG1	None	4µg/mL	Abcam
CSF-1	ROS 5H11-4	Mouse monoclonal anti-porcine	IgG2a	None	10µg/mL	A gift from the Roslin Institute Immunological Toolbox
CSF-1-r	ROS 8G11-1	Mouse monoclonal anti-porcine	IgG2a	None	10µg/mL	A gift from the Roslin Institute Immunological Toolbox
ADGRE1	ROS 4E12-3E6	Mouse monoclonal anti-porcine	IgG1	None	10µg/mL	A gift from the Roslin Institute Immunological Toolbox
Bu-1	AV20	Mouse monoclonal anti-chicken	IgG1 isotype control	None	10µg/mL	R&D systems
KLH	20102	Mouse monoclonal anti-human	IgG2a isotype control	None	10µg/mL	R&D systems
KLH	20102	Mouse monoclonal anti-human	IgG2a isotype control	AlexaFluor647	10µg/mL	R&D systems
IgG (H+L)	Ab150113	Goat polyclonal Anti-mouse	Secondary antibody	AlexaFluor488	5µg/mL	Abcam

The method for dual-colour staining was similar to that described above for single-colour staining, except that two isotype mis-matched primary antibodies were added to each sample (Table 11.1). Additionally, sequential wells of cells were stained with a single one of each of the fluorochromes used to act as compensation controls. Analysis of all stained cells was carried out using the BD Fortessa X20 flow cytometer and FlowJo software; 10,000 events (live cells) were collected. A representative gating strategy is demonstrated in Figure 11.4.

11.2.5 The Comparative Efficacy of Recombinant Canine, Porcine and Human CSF-1 for Generating Canine Macrophages

A previous study has demonstrated that circulating canine and feline monocytes are capable of maturation into macrophage-like cells when grown *in vitro* in culture media supplemented with 10^4IU/mL of recombinant human (rh)-CSF-1 (Gow *et al.*, 2013; Heinrich *et al.* 2017). Therefore, the same concentration (a gift from Professor David Hume) was initially used here to determine if macrophage cells could be derived from canine bone marrow. The resulting cells were assessed as being macrophages based on their morphology by light microscopy, expression of macrophage-associated cell surface molecules during the differentiation time course (Section 11.2.4) as well as adherence to treated tissue culture plastic and phagocytic capacity at the culmination of differentiation.

The biological efficacy of recombinant canine (rc) and recombinant porcine (rp) CSF-1 (gifts from Dr Lissa Herron, The Roslin Institute) was assessed by testing cell viability via MTT (3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) assay. In this assay the relative activity of NAD(P)H-dependent cellular oxidoreductase enzymes reflects the number of viable cells present in the well of a 96-well cell culture plate. These enzymes are capable of reducing the tetrazolium dye MTT to its insoluble formazan, which has a purple colour.

Bone marrow was recovered from cryopreservation and viable cells (determined by Trypan blue staining) were diluted to a concentration of $2x10^7$ cells/mL in complete culture media before 100µL was added to each well of a 96-well cell plate (Nunc™; Thermo Fisher Scientific, USA) *i.e.* $2x10^6$ cells were added per well. A serial two-fold dilution series of each r-CSF-1 were made in complete culture media, starting at a concentration of 2000ng/mL and ending at 32ng/mL. A negative control condition was included by adding 100µL of complete culture media without r-CSF-1 supplementation to triplicate wells of bone marrow cells from each dog. A positive control condition was included by the addition of rh-CSF-1 diluted to a final concentration of 10^4IU/mL in complete culture media to triplicate wells of bone marrow cells

from each dog. The remainder of the cells had 100µL of the CSF-1 dilutions added in triplicate so that the final concentration of cells was $1x10^6$/well.

Plates were incubated at 37ºC/5% CO_2 for ten days with media changes (as described above in section xx) and the addition of fresh growth factor at Day 3 and Day 6. At Day 10 the media was removed and discarded, following this 100µL of RPMI-1640 media containing MTT at a final concentration of 1mg/mL was added to each well. The plates were returned to the incubator for three hours. At the end of the incubation period the MTT containing media was aspirated and discarded. To solubilise the formazan produced, 150µL of 4mM HCl in isopropanol was added to each well. The plate was covered in aluminium foil to protect the contents from light and incubated at room temperature on a plate shaker for 30 minutes. At the end of the 30 minutes the plate was visually checked to ensure that all of the formazan was in suspension, if not then the plate was returned to the plate shaker and rechecked at 10 minute intervals until this was achieved.

The optical density was quantified by measuring the absorbance at OD570nm; the data was exported to Microsoft Excel and analysed using GraphPad Prism 7.

11.2.6 Phagocytosis Assay

Following harvest of adherent BMDM cells after 10 days of differentiation in CSF-1 supplemented complete culture media, $1x10^6$ cells/ml were plated per well of a 6-well tissue culture plate in duplicate and cultured overnight at 37°C/5% CO_2. Phagocytosis was initiated by the addition of fluorescein isothiocyanate (FITC)-conjugated Zymosan bioparticles (Molecular Probes, USA) at a particle to cell ratio of approximately 5:1, followed by further incubation for one hour. A negative control well was included where PBS was added to the complete culture media without Zymozan particles. Phagocytosis was stopped by the addition of 500 µL/well of ice-cold sterile PBS, followed by two further washes in ice-cold sterile PBS to remove extracellular particles.

All cells were fixed with the addition of 2% paraformaldehyde solution for 30 minutes at room temperature. The culture wells were washed twice with sterile PBS before 1mL of 0.1% Triton X100 solution (Sigma Aldrich, UK) in PBS was added for five minutes at room temperature in order to permeabilise cells. Blocking buffer comprising 3% bovine serum albumin in PBS was added to the cell monolayer and incubated at room temperature for one hour. After this incubation, blocking buffer was replaced with a solution of phalloidin Texas Red™ (Molecular Probes, USA) diluted 1:500 by volume in blocking buffer and incubated in the dark at room temperature for one hour.

The cell plates were washed twice with PBS. Nuclear material was counterstained with the addition of 300nM 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes, USA) solution for five minutes at room temperature. The wells were washed five times with 1mL deionised water which was not removed after the final wash. Cells were analysed for Zymosan particle uptake using fluorescence microscopy (Zeiss LSM710).

11.2.7 Culture of Mycobacterial Reference Strains

Reference strains of *Mycobacterium (M.) bovis* bacile Calmette–Guérin (*M. bovis*-BCG, strain 1173P2 [Pasteur]) and *M. bovis* (strain ATCC BAA-935 / AF2122/97) were grown in the containment level two and three laboratory facilities of The Roslin Institute, University of Edinburgh, respectively. Single aliquots containing 1mL of mycobacteria suspended in culture media were added to 9mL of Middlebrook 7H9 media (Sigma Aldrich, UK) supplemented with Middlebrook ADC growth supplement (Sigma Aldrich, UK) to 10% by volume and 0.5g/500mL Tween 80 (Sigma Aldrich, UK).

Cultures were incubated at 37°C/5% CO_2 with agitation in a shaking incubator for 14 days, after this time the density was measured daily until an OD_{600} of 0.6 was reached, indicating that the mycobacteria had reached the exponential growth phase. At this time, the 10mL culture was added to a further 90mL of Middlebrook 7H9 ADC media and returned to the incubator. Again, the cultures were grown 37°C/5% CO_2 with agitation for 14 days before daily

assessment of the OD value. Once an OD_{600} of 0.6 had been reached, the mycobacteria were aliquoted into sterile 1mL screw-topped Eppendorf tubes and stored at -80 ^{o}C until further use.

Quantification of mycobacteria was conducted throughout by assessing the colony forming unit (CFU) count. To achieve this, serial ten-fold dilutions of thawed bacterial stock culture, or infected macrophage lysate (Section 11.2.8), were carried out in Middlebrook 7H9 ADC to a dilution factor of $1x10^{-6}$. Once diluted, 10µL of each dilution was pipetted in duplicate onto Middlebrook 7H11 solid agar plates supplemented with Middlebrook OADC mycobacterial growth supplement (Sigma Aldrich, UK). Plates were incubated upside down at 37ºC/5% CO_2 for 16 days. Colony numbers were counted at the dilution factor which allowed between 15 and 50 colonies to be counted to maintain accuracy. The duplicate values were averaged and the dilution factor was used to calculate the original CFU/mL in the starting sample.

11.2.8 Infection of Bone Marrow Derived Macrophages with Mycobacteria

After 10 days in CSF-1 supplementes culture the resulting BMDM were detached from tissue culture plates by removing and discarding the culture media and washing the cell monolayer twice with PBS to remove serum remnants; following this, 10mL of non-enzymatic cell dissociation solution (Sigma Aldrich, UK) was added to each flask and incubated at 37ºC until the cells visibly detached from the surface of the plastic as assessed using an inverted light microscope. The cell suspension was removed from the plates into 50mL Falcon tubes, centrifuged at 600 x *g* for five minutes and the supernatant was removed and discarded. Cell pellets were re-suspended in complete culture media containing r-CSF-1 at concentrations with equivalent biological activity to 10^4U/mL of rh-CSF-1 (as established in Section 11.2.5) and cells were diluted to a concentration of 10^6 cells/mL. A single mL of cell suspension was added to each well of a 6-well treated tissue culture plate and incubated at 37ºC/5% CO_2 for 16-24 hours to allow the cells to re-adhere to the plastic.

Aliquots of mycobacterial strains, frozen at -80ºC, were thawed at room temperature and diluted in complete culture media to a concentration that would give a defined multiplicity of infection (MOI) per mL. Media was removed from cell culture monolayers and replaced with

1mL of the mycobacterial suspension. Media was removed from uninfected control wells and replaced with 1mL warmed (37ºC) complete culture media.

Plates were centrifuged at 600 x *g* for five minutes, at room temperature, and then incubated at 37ºC/ 5% CO2. At each experimental timepoint, mycobacteria-containing media was removed from each well and macrophage monolayers were washed three times with warmed sterile PBS (37ºC) to remove extra-cellular bacteria. All control wells were treated the same way. The PBS was replaced with 1mL per well of sterile cell culture media and plates returned to the incubator for the remainder of the infection time-course and samples were taken for downstream processing at the appropriate timepoints.

For cells stimulated with LPS (Section 11.2.10) or infected with mycobacteria (Section 11.2.8), cell culture supernatants were harvested at various time points post-stimulation and frozen at -80ºC before analysis by ELISA (Section 11.2.11) and Griess assay (Section 11.2.12). For analysis by reverse-transcription quantitative PCR (RT-qPCR; Section 11.2.13) cells were lysed in RNA buffer (RNA ReliaPrep System; Promega, USA) and stored frozen at -80ºC until further analysis.

11.2.9 Culture of DH82 Cells

The cell line, DH82 (CRL-10389; American Type Culture Collection [ATCC], USA), represents a macrophage-like immortal cell lineage derived from a spontaneously arising histiocytic sarcoma of a 10-year old Labrador in 1988 (Wellman, 1988). Upon receipt of the cell line (on dry ice) from ATCC, the vial was defrosted in a water bath pre-warmed to 37ºC. The thawed cells were transferred to a 15mL Falcon and 10mL of warmed complete culture media was added dropwise with agitation. The cells were centrifuged at 600 x *g* for five minutes and the supernatant was removed. Cells were re-suspended in 20mL of complete culture media, added to a 75cm^2 treated tissue culture flask (Nunc^{TM}; Thermo Fisher Scientific, USA) and incubated at 37ºC/5% CO_2. After 24 hours the media was removed (along with any non-adherent cells) and the cell layer was washed twice with warmed (37ºC) sterile PBS; 20mL of fresh complete culture media was added and the flask was returned to the incubator. After

three days, when the cells reached approximately 80% confluence, the media was removed and the cell layer was washed twice with PBS before 10mL 0.25% Trypsin-0.53mM EDTA solution (Sigma Aldrich, UK) was added to the flask and incubated at 37°C until the cells visibly detached from the plastic. A count of viable cells was performed by mixing 10µL of cell suspension with the same volume of 2% sterile filtered Trypan blue and assessed using a haemocytometer. The cells were centrifuged at 600 x *g* for five minutes and the supernatant discarded. The cell pellet was then re-suspended in complete culture media to a density of $1x10^4$ cells/mL and 20mL were added to one or more $75cm^2$ treated tissue culture flasks (Nunc™, Thermo Fisher Scientific, USA) as required and returned to the incubator. Remaining cells were cryopreserved to maintain low passage number cells; all experiments were performed on cells between passage number two and five. Complete culture media was replaced every three days between sub-cultures.

11.2.10 Stimulation of Bone Marrow Derived Macrophages with Lipopolysaccharide

On day 10 of differentiation in r-CSF-1, BMDM were detached from tissue culture plates (Section 11.2.3) by removing and discarding the culture media, washing the cell monolayer twice with PBS to remove serum remnants. Following this, 10mL of non-enzymatic cell dissociation solution was added to each flask and incubated at 37°C until the cells visibly detached from the surface of the plastic as assessed using an inverted light microscope. The cell suspension was removed from the plates into 50mL Falcon tubes, centrifuged at 600 x *g* for five minutes and the supernatant was removed and discarded. Cell pellets were re-suspended in complete culture media containing r-CSF-1 at concentrations with equivalent biological activity to 10^4U/mL of rh-CSF-1 and cells were diluted to a concentration of 10^6cells/mL.

A single mL of cell suspension was added to each well of a 6-well treated tissue culture plate and incubated at 37°C/5% CO_2 for 16-24 hours to allow the cells to re-adhere to the plastic. The cells were treated with LPS from *Salmonella enterica* serotype Minnesota strain 595 (Re

mutant) (Sigma-Aldrich, UK) at a final concentration of 100 ng/ml other than one well in each plate where the media was replaced with fresh complete culture media to act as an unstimulated control well. Stimulations were run in duplicate for each condition assessed.

11.2.11 Quantification of Cytokine Secretion by ELISA

The cytokines TNF-α (DY1507) and IL-6 (DY1609) were quantified by commercial canine ELISA purchased from R&D Systems (United States). For each assay, flat bottomed 96-well plates (Nunc Maxisorp^{TM} plates; Sigma Aldrich, UK) were coated with the appropriate capture antibody diluted in carbonate/bicarbonate buffer or PBS at the concentration instructed and incubated overnight at room temperature.

Wash steps were conducted as follows: plates were washed five times in PBS/ 0.05% Tween-20, using the Skan Washer 400 plate washer. Coated plates were washed and blocked for between one and two hours using 100µL per well of Reagent Buffer (R&D Systems; United States). Plates were washed again, and 50µL of supernatants were added to the plate in duplicate. Recombinant cytokine was serially diluted 1:2 (in duplicate) to produce a standard curve, and the plate was incubated at room temperature for one hour. A wash procedure was carried out, and 50µL of the appropriate biotinylated detection antibody was added to each well and incubated at room temperature for one hour. Plates were washed and 50µL streptavidin-HRP (provided with the ELISA kit, diluted 1:200 in reagent diluent as instructed) was added to each well. Plates were incubated for one hour then washed again. Subsequently, 50µL of TMB substrate solution (1-Step TMB Ultra ELISA Substrate; Thermo Fisher Scientific, United States) was added to each well and plates were incubated in the dark until colour appeared in the bottom standard. The reaction was stopped by the addition of 25µL 1M H_2SO_4 per well. Absorbance was measured at 450nm using the Synergy HT Multi-Mode Microplate Reader and Gen 5 software. The results were exported to Microsoft Excel and subsequent analysis was performed in GraphPad Prism 7.

11.2.12 Quantification of Nitrite by Griess Assay

Following differentiation, BMDM were stimulated with LPS (as described in Section 11.2.10) or were infected with mycobacteria (as described in Section 11.2.8). The concentration of nitrite produced was measured in the supernatant at each time point of stimulation or infection using the Griess Reagent System (Thermo Fisher Scientific, US). Initially, a seven-point two-fold serial dilution of nitrite standard diluted in RPMI 1640 culture media was produced ranging from 100µM to 1.56µM and 50µL of each concentration was plated in triplicate into a 96-well tissue culture plate (Thermo Fisher Scientific, United States). The sulfanilamide and *N*-1-napthylethylenediamine dihydrochloride (NED) solutions were left for 15-30 minutes to equilibrate to room temperature. During this time 50µL of each experimental sample was plated in triplicate in the remainder of the plate. Once equilibrated, 50µL of the sulfanilamide solution was added to all experimental samples and wells containing the dilution series for the nitrite standard reference curve. The plate was then incubated for between five and ten minutes at room temperature, protected from light, before 50µL of the NED solution was added to all wells. The plate was again incubated at room temperature for between five and ten minutes, protected from light. Once the purple/magenta colour had formed in the wells containing the lowest concentration of nitrite standard the absorbance was measured at 535nm, using the Synergy HT Multi-Mode Microplate Reader and Gen 5 software.

11.2.13 Quantification of Cytokine Gene Expression by RT-qPCR

Cells challenged with either LPS or mycobacteria were collected in 500µL RNA buffer (Buffer BL+TG; Promega ReliaPrep™ RNA Cell and Tissue Miniprep System) which was stored frozen at -80^{o}C until needed. The RNA was extracted by thawing the cell lystate, adding 170µL isopropanol to each sample and vortexing each before transferring the solution to a Promega ReliaPrep™ Minicolumn. The columns were centrifuged at room temperature at 6,000 x *g* for 30 seconds and the flow through was discarded. The RNA was washed sequentially with 500µL of RNA Wash Buffer 1 and 2 (Promega ReliaPrep™ RNA Cell and Tissue Miniprep System) with the columns being centrifuged at 6,000 x *g* for 60 seconds between each wash. The gDNA was removed from the samples by the addition of 30µL of DNase I incubation mix

(Promega ReliaPrep™ RNA Cell and Tissue Miniprep System) to each column for 15 minutes at room temperature. Each column was washed sequentially with 300µL of Column Wash Buffer and twice with 500µL of RNA Wash Buffer 1; columns were centrifuged at 6,000 x *g* for 60 seconds at room temperature between washes. The RNA was eluted into 50µL of nuclease-free water and the quantity of RNA was measured using spectrophotometry (Nanodrop™).

For each sample, 1µg of cDNA was synthesised using the GoScript™ Reverse Transcription System Protocol (Promega, USA) according to the manufacturer's instructions. For each sample a reaction was run which did not include the reverse transcriptase enzyme (RT negative controls). The RT negative controls were evaluated for gDNA contamination by attempting to amplify the constitutively expressed reference gene (*i.e.* a housekeeping gene) *HPRT1.* For each sample a PCR reaction mixture of 5µL of GoldStar PCR Mix (Eurogentec, Belgium) was added 0.25µL of 25 pmol/mL oligonucleotide primer mix, 0.15mL of $MgCl_2$, 1µL of sample as template and 3.6µL nuclease-free H_2O was prepared. The PCR protocol consisted of heating to 95°C for three minutes followed by 25 cycles of heating to 95°C for 30 seconds, cooling to 60°C for 30 seconds and warming to 72°C for 45 seconds before a final step of holding at 72°C for five minutes. The products were analysed by agarose gel electrophoresis using a 3% agarose gel and SYBR Safe stain. Only samples free of gDNA contamination were used for quantitative PCR (qPCR) analysis.

The DNA sequences of candidate reference genes and the genes of interest were obtained from the NCBI nucleotide database and copied into the input window of the Primer3 website (http://primer3.ut.ee/). The product size range was set to between 80 and 150 base pairs. The number of primer pairs to be returned was set to ten and the list was rigorously checked in order to only select primer pairs that would result in minimal complementarity and therefore limit primer dimer formation. Where possible, primer pairs were selected which spanned an exon-exon junction and A BLAST search was run to ensure the primer pairs would not anneal to any non-target DNA sequences. Two primer pairs were tested per reference gene and details of all primers used are detailed in Table 11.2

Table 11.2: Canine RT-qPCR primers used in this study. All sequences are provided 5' to 3'; bp: base pairs

Target Gene	Forward Primer	Reverse Primer	Product Size (bp)
Reference Genes			
GAPDH (1)	GTGAACGGGAAGCTCACTGG	TCCGATGCCTGCTTCACTAC	131
GAPDH (2)	GCTGAACGGGAAGCTCACT	CGATGCCTGCTTCACTACCT	130
β-Actin (1)	TCCTTCCTGGGCATGGAATC	GAGGTCCTTGCGGATGTCAA	87
β-Actin (2)	CTTCCTGGGCATGGAATCCT	CCAGGGTACATGGTGGTTCC	129
HPRT-1 (1)	CTTCTGCAGGAGAACCTCCG	TCACTAATCACGACGCTGGG	125
HPRT-1 (2)	CTGGCTATCATTGATCAGGTGA	AACAAGCTCTGGCCAGTTTTA	112
RPS-7 (1)	CAAGATCGTGAAGCCCAACG	ACTTCAATCTCCTTGGCCGC	141
RPS-7 (2)	TGAACTCCGACCTCAAAGCC	CCGCCAACTTCAATCTCCTTG	80
RPL-17 (1)	TCTTTCGGGAGGAAATCTCGC	ACCTCTCGACTTGCATGATTTTG	98
RPL-17 (2)	GATCACGTTCTTTCGGGAGGA	CCTCTCGACTTGCATGATTTTGT	102
RPL-30 (1)	TCTGCATTGTGGGAGCTCTTT	CTCCAGCGACTTTTTCGTCTT	125
RPL-30 (2)	GGAAGAGCTCTGCATTGTGG	TCCAGCGACTTTTTCGTCTTCT	130
Genes of Interest			
MHC-II	ACCTTTCTGGCTCCTGTGAC	GGACAACTCTGACTCTCAGGC	146
IL-1β	CCTAGGAATGGGGCCTTCATC	TGCATGCCAGCTACTACACC	145
CYP27B1	CTAACCTGCAACCCTCTCCT	GGGATTTCTTGCCTCGGCTC	148
CCL20	GGACATTTGCCATTGGCTCC	GGCATAAGGGTCCCAGTGAG	80
IDO	CTGTCCAGCAACTCCAGTCC	AGAGACAGGTGAGAGTGTCTGA	91
NOS2	CTGGCCCTTCTCAATATGGCT	CAAGCACAGATGAAGTGGGTG	117

For each gene, a 7-point standard curve of 10-fold dilutions of cDNA was conducted in order to calculate the efficiency of the reactions and optimise the primer and cDNA concentrations used. All qPCR reactions were performed using Takyon™ ROX SYBR 2X Mastermix (Eurogentec, Belgium). Master mixes were made for each gene transcript as follows; 5.0µL 2X Takyon mix, 1µL 25pmol mixed forward and reverse primer solution and 1.5µL nuclease-free H_2O.

A total of 7.5µL of a single master mix was dispensed into each well of a 96 well plate, on ice, and 2.5µl of cDNA (diluted to the optimum concentration in nuclease-free H_2O) or non-template control added to appropriate wells. Two reference gene transcripts were evaluated contemporaneously to the genes of interest. Each sample to be tested was represented in duplicate. Wells were sealed with Ultra Clear qPCR caps and the plate was centrifuged at 400 x *g* for two minutes. The reaction was run on a Stratagene Mx3000P qPCR machine under

the following conditions; one cycle of heating to 50°C for two minutes and 95°C for two minutes followed by 45 cycles of heating to 95°C for 15 seconds followed by colling to 62°C for 30 seconds a final single cycle of heating to 95°C for 60 seconds, cooling to 62°C for 30 seconds and heating to 95°C for 30 seconds whilst the dissociation curve was recorded. The dissociation curve was included in the protocol to ensure that only a single product was generated. All RT-qPCR data was analysed using MxPro software and Microsoft Excel. Relative quantification of the target transcript compared to the reference transcript was calculated using the $2^{-\Delta\Delta Ct}$ method to determine fold-change in gene expression normalised to both of the included reference genes.

11.3 Results

11.3.1 Bone marrow cells harvested from dogs differentiate into macrophage-like cells in response to rh-CSF-1

Previous studies have shown that rh-CSF-1 readily differentiates murine femoral bone marrow cells into mature macrophages and more recently the same has been shown for pigs (Kapetanovic *et al.* 2012; Assouvie *et al.* 2018). In both of these species, bone marrow cells can be cryopreserved and successfully differentiated upon recovery, for pigs this means that many cells can be obtained from the same animal and so this reduces the overall number of animals required for research purposes.

In dogs, a number of culture conditions can induce the maturation of circulating monocytes into macrophages which are adherent to bacteriological plastic tissue culture dishes (Bueno *et al.* 2005). However, the acquisition of monocytes from dogs *ante mortem* is ethically difficult as it requires an invasive procedure (venepuncture and blood collection) and the number of cells obtainable is limited by the size of the dog (and therefore the quantity of blood that it is safe to draw). In addition, only a relatively small percentage of canine circulating peripheral blood mononuclear cells (PBMC) are monocytes; typically 2-4%, compared, for example, to bovine PBMC which are 10-15% monocytes.

The first aim of this study was to develop a protocol for the efficient removal of bone marrow cells from the long bones of dogs, to maintain cell viability following cryopreservation and recovery of these cells, and to differentiate bone marrow cells into mature bone marrow derived macrophages (BMDM).

Bone marrow was isolated from three dogs (Table 11.3) which yielded between $1.4x10^8$ and $1.2x10^9$ cells per animal. Cells were either cryopreserved or plated immediately with the addition of 10^4IU/mL rh-CSF-1 (Figure 11.1) as this concentration has previously been shown to be effective for the generation of porcine BMDM and for maturing canine monocytes into mature macrophages (Kapetanovic *et al.* 2012).

Table 11.3: Signalment details of the dogs used to obtain bone marrow for the initial BMDM culture protocol optimisation.

Dog Number	Breed	Age (years)	Sex
Dog 1	Jack Russel Terrier	Five	Male Neutered
Dog 2	Staffordshire Bull Terrier	Two	Male Entire
Dog 3	Pitbull Terrier	One	Male Entire

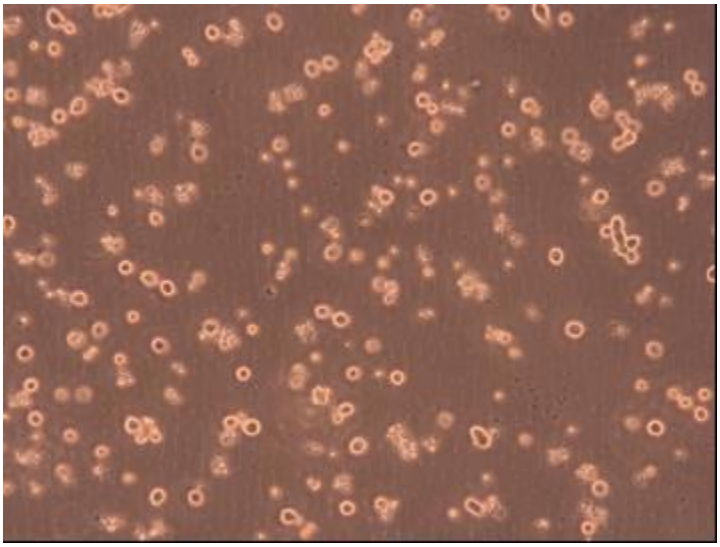


Figure 11.1: Bone marrow cells from Dog 1 plated at 10^6 cells/mL on Day 0 in complete culture media supplemented with 10^4IU/mL rh-CSF-1. Representative image from one dog.

Cells were assessed regularly until the majority had become adherent to the bacteriological plate and displayed the approximately round, vacuolated morphology of a mature macrophage. For all three dogs, this was found to be greatest after ten days in culture as shown in Figure 11.2.

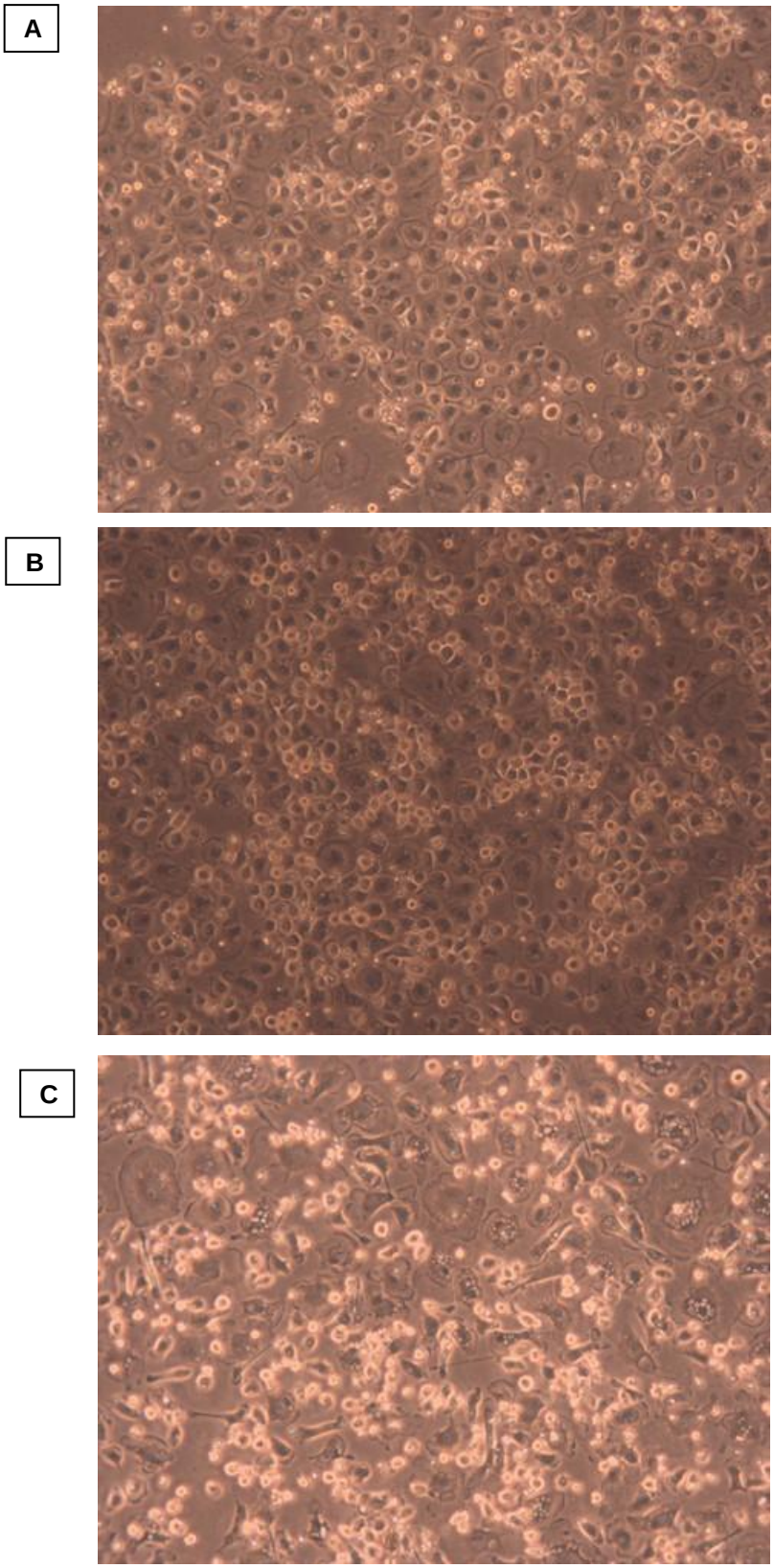


Figure 11.2: Representative images of BMDM cells from Dog 1 [A], Dog 2 [B] and Dog 3 [C] after ten days in complete culture media supplemented with 10^4IU/mL rh-CSF-1. Images taken at x40 magnification.

Following cryopreservation, the viable cell recovery rate was between 63% and 80%. Thawed cells differentiated to produce morphologically identical cells to when bone marrow cells were plated from fresh (Figre 11.3); therefore future experiments used only cryopreserved cells.

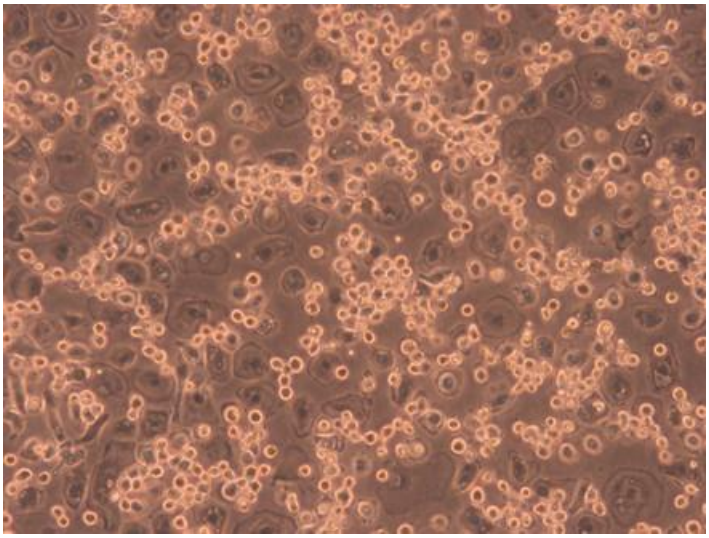


Figure 11.3: Representative image of bone marrow derived macrophage cells from Dog 1. The bone marrow cells were initially cryopreserved, thawed and cultured for ten days in complete culture media supplemented with 10^4IU/mL rh-CSF-1. Image taken at x40 magnification.

11.3.2 Canine bone marrow cells cultured in rh-CSF-1 acquire cell surface molecules typical of macrophages within ten days

Human circulating monocytes are frequently subdivided into classical, intermediate and non-classical phenotypes based on cell surface CD14 and CD16 expression (reviewed by Ziegler-Heitbrock and Hofer, 2013) whereas pig monocytes can be grouped by CD16 and CD163 expression (Ziegler-Heitbrock, 2014). The surface molecule CD172a (SIRPα) is most associated with expression on myeloid cells whilst ADGRE-1 (F4/80) is a widely used molecule specific to murine macrophages (Bastos *et al.* 2007; Waddell *et al.* 2018). The aim of this study was to use flow cytometric analysis of these and other cell surface molecules (CSF-1 and the CSF-1 receptor [CSF-1r]) to characterise the surface expression profile of canine bone marrow cells, as well as to examine and monitor changes in their expression profile during macrophage

differentiation in rh-CSF-1. The flow cytometry methodology (including antibodies used) is provided in Section 11.2.4.

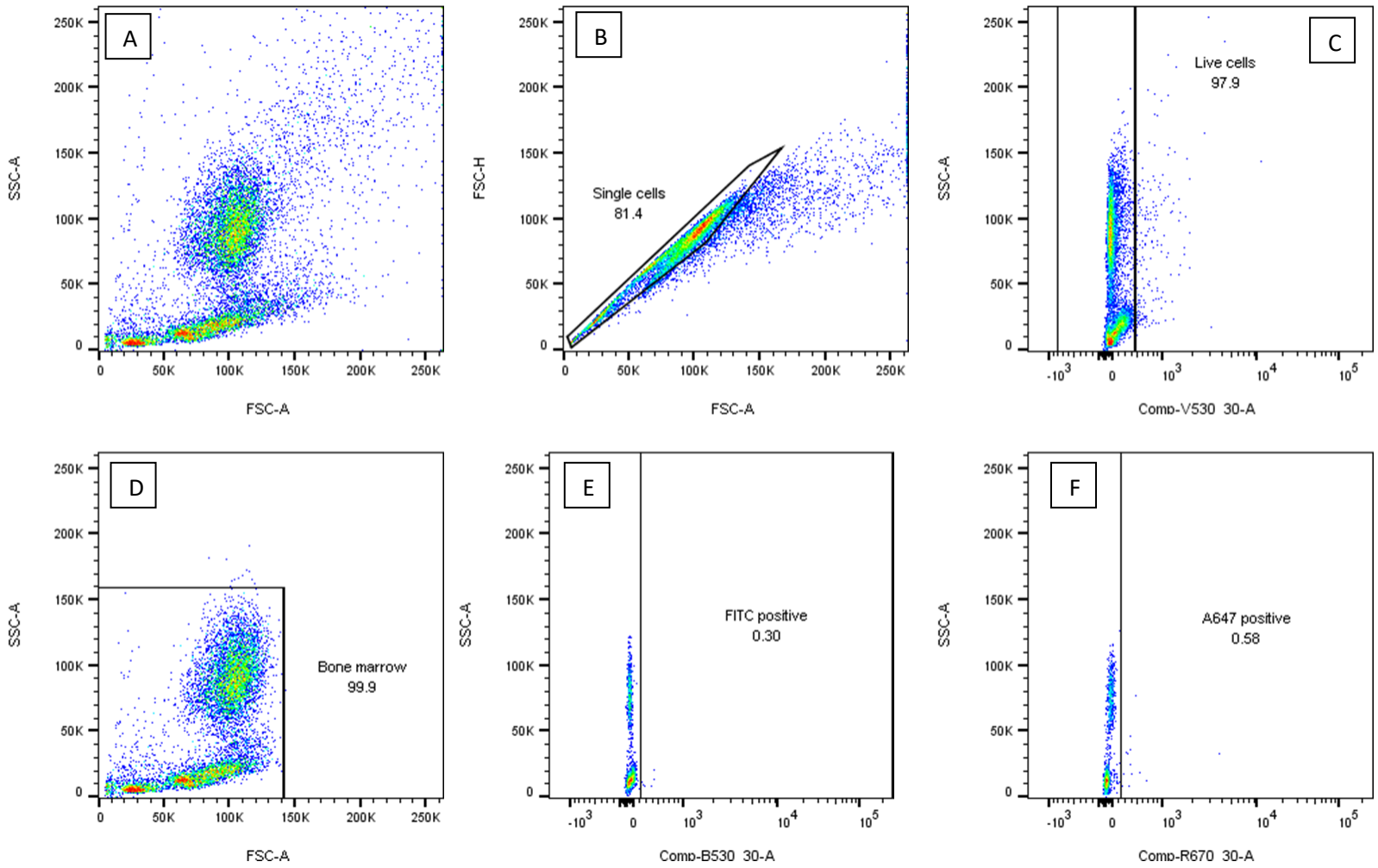
Bone marrow from three dogs (Table 11.4) was assessed at four timepoints; Day 0, Day 3, Day 6 and Day 10 of differentiation and an example gating strategy as applied to all samples is shown in Figure 11.4. The size and granularity of the cells was assessed by comparing the side scatter against forward scatter profiles; these cytometry plots from each timepoint for Dog 4 are shown in Figure 11.5 as a representative example of the changes seen for all animals. The percentage of cell surface molecule-positive cells at each time point for all three animals tested is shown in Table 11.5 whilst individual plots for the single-colour staining are displayed in Figure 11.6.

Table 11.4: Signalment details of the dogs used to obtain flow cytometry data during the differentiation of BMDM cells.

Dog Number	Breed	Age (years)	Sex
Dog 3	Pitbull Terrier	One	Male Entire
Dog 4	Staffordshire Bull Terrier	Three	Female Entire
Dog 5	Unknown Cross	Four	Female Entire

Table 11.5: The percentage of cells expressing each of the cell surface molecules tested for each of the four time points measured. **N.B.** The anti-CSF-1 receptor antibody tested did not label any canine cells at any time point.

Cell surface molecule	Day 0	Day 3	Day 6	Day 10
Dog 3				
CSF-1	87.2.0	22.7	53.4	97.0
ADGRE-1	3.64	8.02	11.2	87.5
CD172a	59.9	23.1	58.9	97.2
CD163	11.8	24.2	67.3	94.7
CD16	47.3	6.88	18.8	56.2
CD14	58.7	35.6	57.5	95.7
$CD14^{hi}$	3.2	6.55	11.5	
CD14/CD172a	54.9	23.0	32.7	98.8
CD14/CD163	4.6	25.9	52.1	100
CD14/CD16	28.2	6.6	12.2	50.0
Dog 4				
CSF-1	59.4	21.2	25.2	96.1
ADGRE-1	3.1	19.8	24.2	91.8
CD172a	68.5	58.5	46.3	96.3
CD163	3.9	17.4	49.8	96.7
CD16	24.8	4.2	9.89	52.0
CD14	56.5	20.1	42.4	98.4
$CD14^{hi}$	3.8	10.1	22.7	
CD14/CD172a	57.8	22.2	33.5	98.2
CD14/CD163	3.4	20.1	47.8	93.1
CD14/CD16	20.2	4.7	11.0	51.2
Dog 5				
CSF-1	56.3	40.2	80.2	96.6
ADGRE-1	5.95	43.5	76.9	95.2
CD172a	52.1	60.7	73.8	99.2
CD163	63.9	39.4	72.7	98.9
CD16	27.4	3.7	41.2	61.4
CD14	47.3	35.8	48.2	98.0
$CD14^{hi}$	4.06	15.8	67.6	
CD14/CD172a	60.3	41.4	44.9	97.2
CD14/CD163	43.2	36.5	48.8	95.3
CD14/CD16	22.0	3.5	7.8	58.4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FSC-A
B
FSC-H
FSC-A
Single cells
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C
SSC-A
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Live cells
97.9
D
SSC-A
FSC-A
Bone marrow
99.9
E
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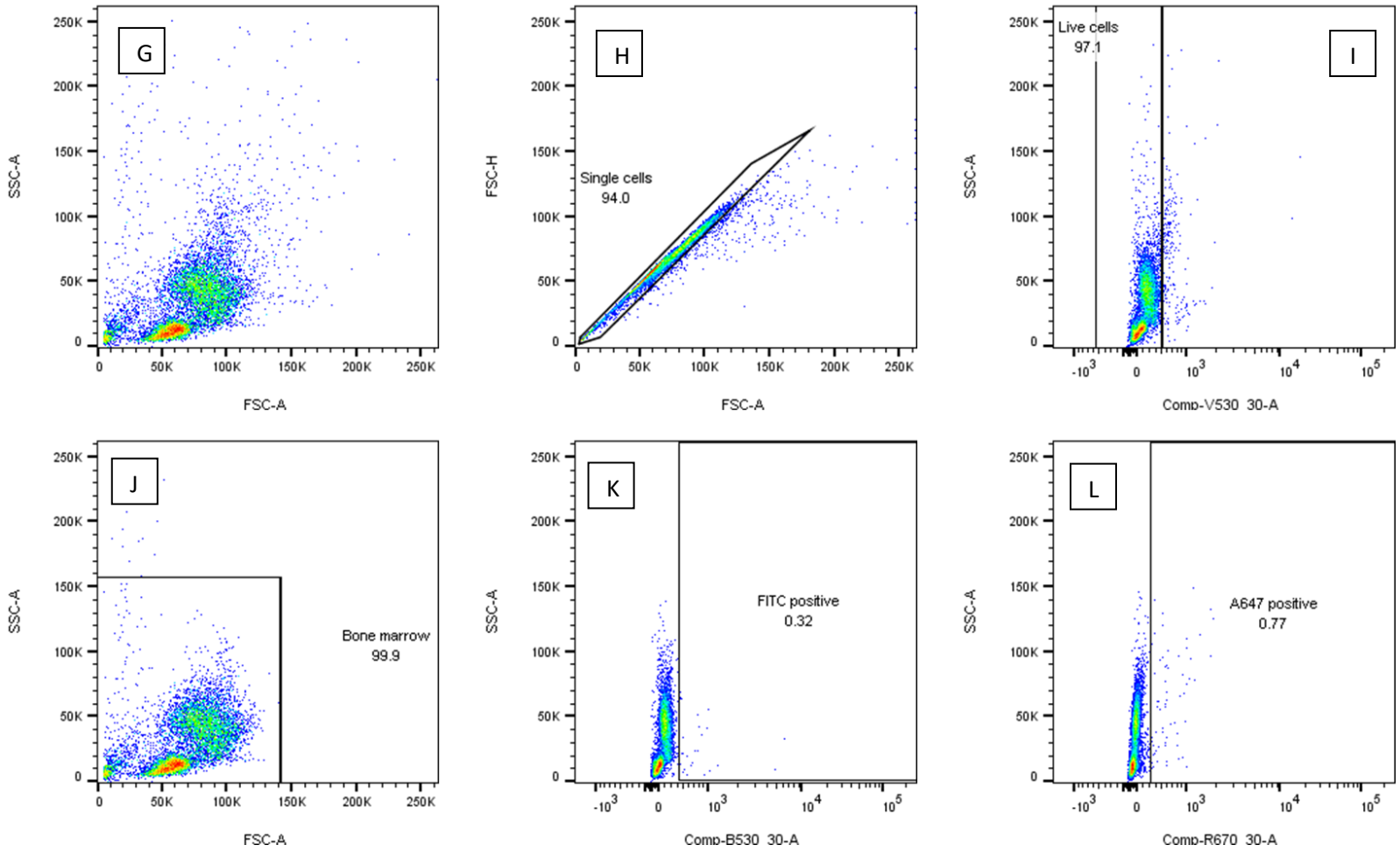
F
SSC-A
Comp-R670 30-A
A647 positive
0.58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FSC-A
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FSC-H
FSC-A
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J
SSC-A
FSC-A
Bone marrow
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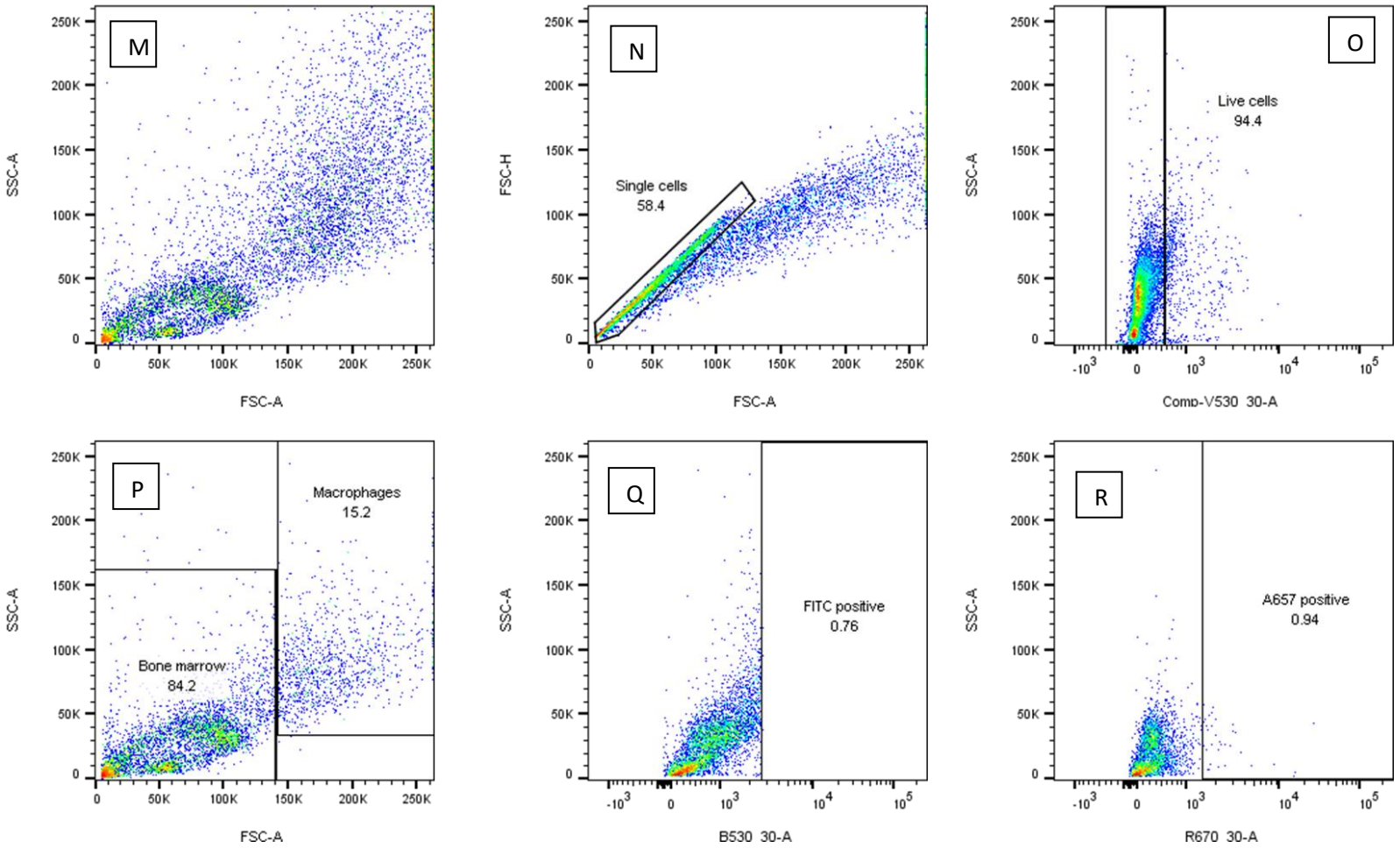
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O
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Live cells
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P
SSC-A
FSC-A
Macrophages
15.2
Bone marrow
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R
SSC-A
R670 30-A
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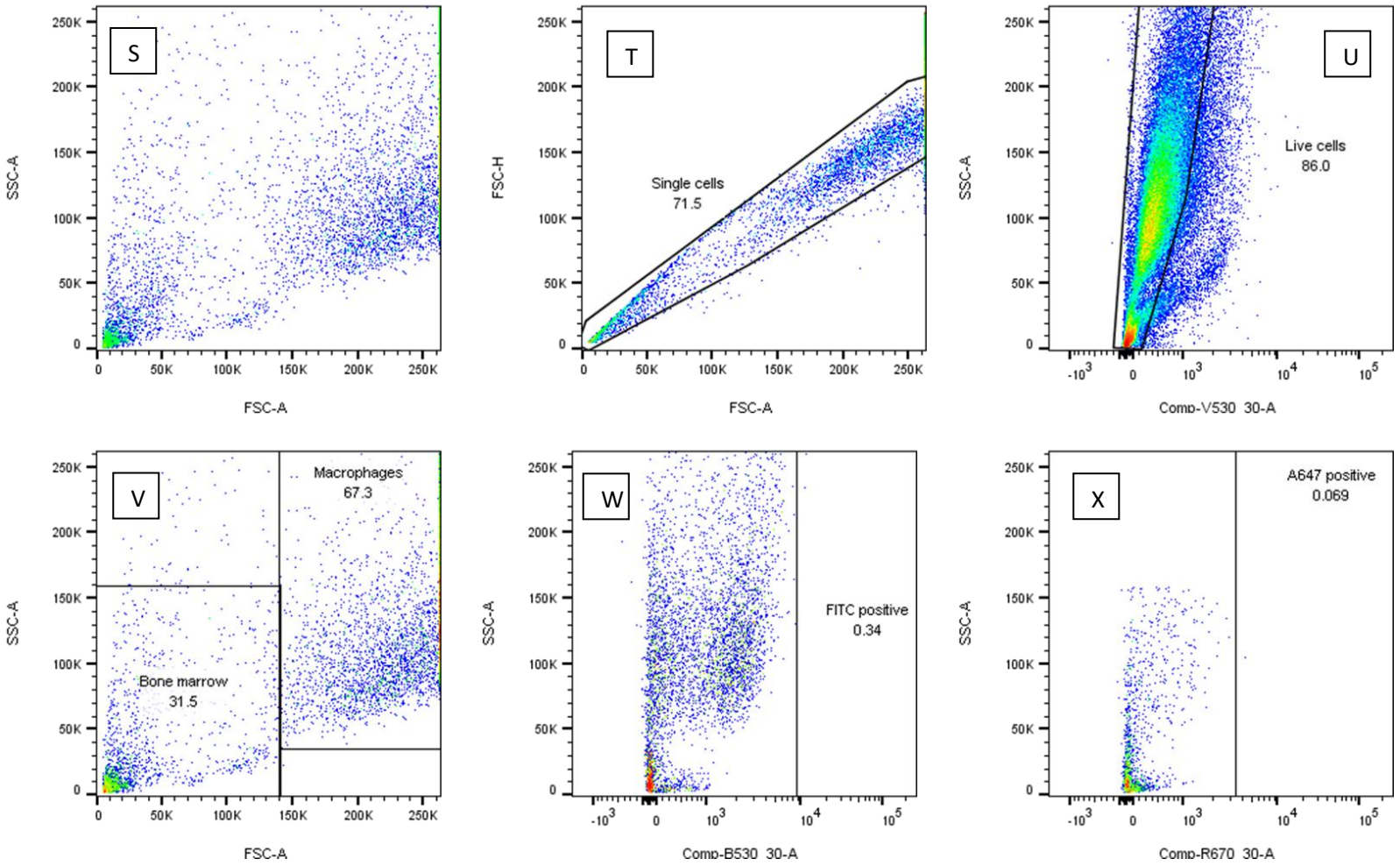
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T
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Single cells
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V
SSC-A
FSC-A
Macrophages
67.3
Bone marrow
31.5
W
SSC-A
Comp-B530 30-A
FITC positive
0.34
X
SSC-A
Comp-R670 30-A
A647 positive
0.069

Figure 11.4: Flow cytometry gating strategy. Representative gating strategy (displaying data from Dog 4) for flow cytometry analysis for day 0 (A-F), day 3 (G-L), day 6 (M-R) and day 10 (S-X). The forward and side scatter of ungated cells are shown for each day in plots A, G, M and S respectively. Forward scatter height (FSC-H) *vs.* forward scatter area (FSC-A) plots (B, G, N and T) were used to reduce the population to single cells only. Dead cells were excluded by gating only on cells negatively stained with the live-dead marker Zombie Violet™ (plots C, H, O and U). The remaing cell populations are shown in plots D, I, P and V with gating applied at 160K SSC-A *vs.* 140 FSC-A to demonstrate the increase in cellular size and granulation over the 10 days of differentiation in r-CSF-1. Plots E, J, Q and W show the gating for unstained cells for in the Blue-530-30 channel (FITC [488nm]) and plots F, K, R and X show the same for the Red-670-30 (AlexaFluor™-647) channel to show the change in auto-fluorescence over the timecourse.

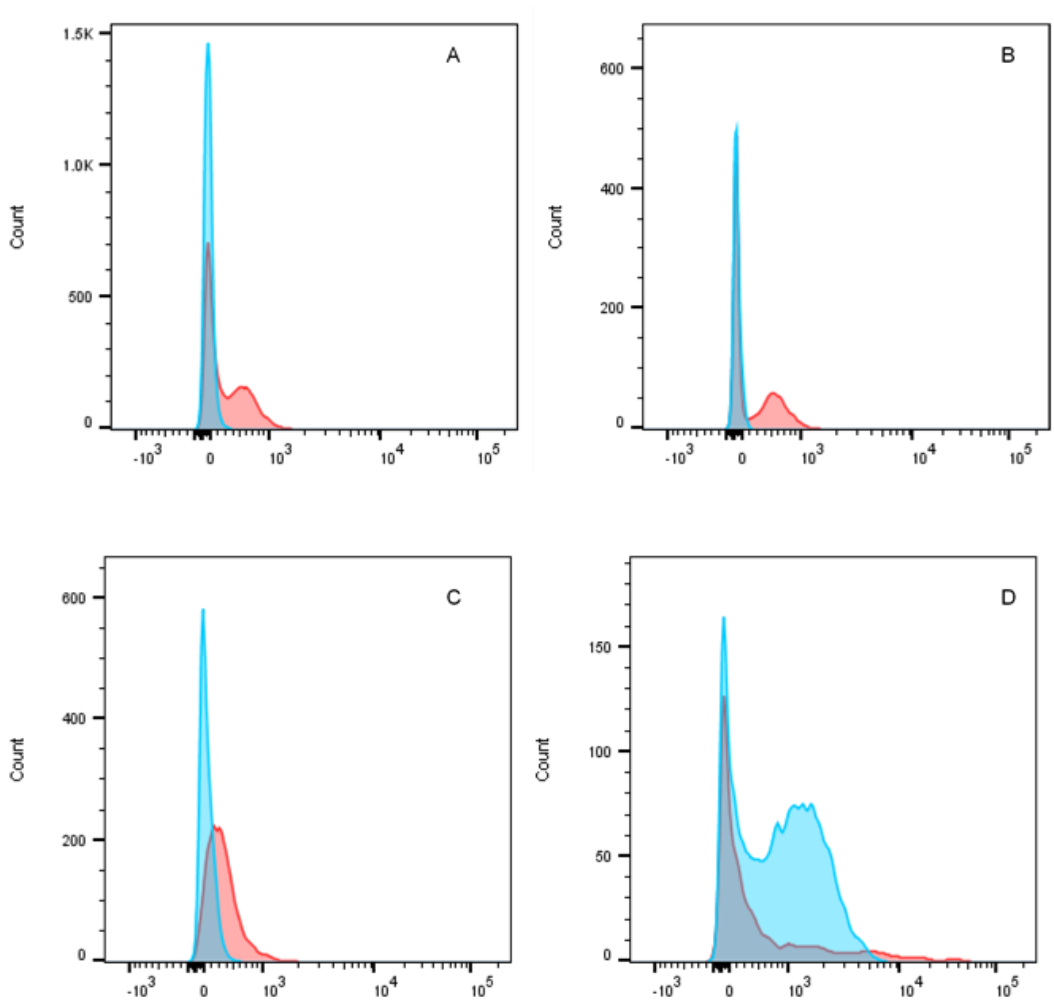
Initially within the total bone marrow cell population there was a large population of mature granulocytes (as determined by size (Fsc) and granularity (SSc): oval gate; Figure 11.4). The bone marrow cells expressed CSF-1, CD14, CD172a and CD16 at Day 0 (Figure 11.5). Based on SSc, the granulocye population expressed CSF-1, CD16 and CD14, and were CD172a low. At Day 0 only a small percentage (3-6%) of the total viable bone marrow cells from each dog expressed ADGRE-1 and CD163.

Over the course of differentiation with rhuCSF-1 the large SSc granulocyte population reduced between Day 0 and Day 3 (Figure 11.4). This was associated with an initial decline in the total percentage of cells expressing CSF-1, CD14, CD172a and CD16 (Figure 11.5). By contrast, the putatively monocyte-macrophage population of cells (lower SSc) that expressed ADGRE-1 and CD163.

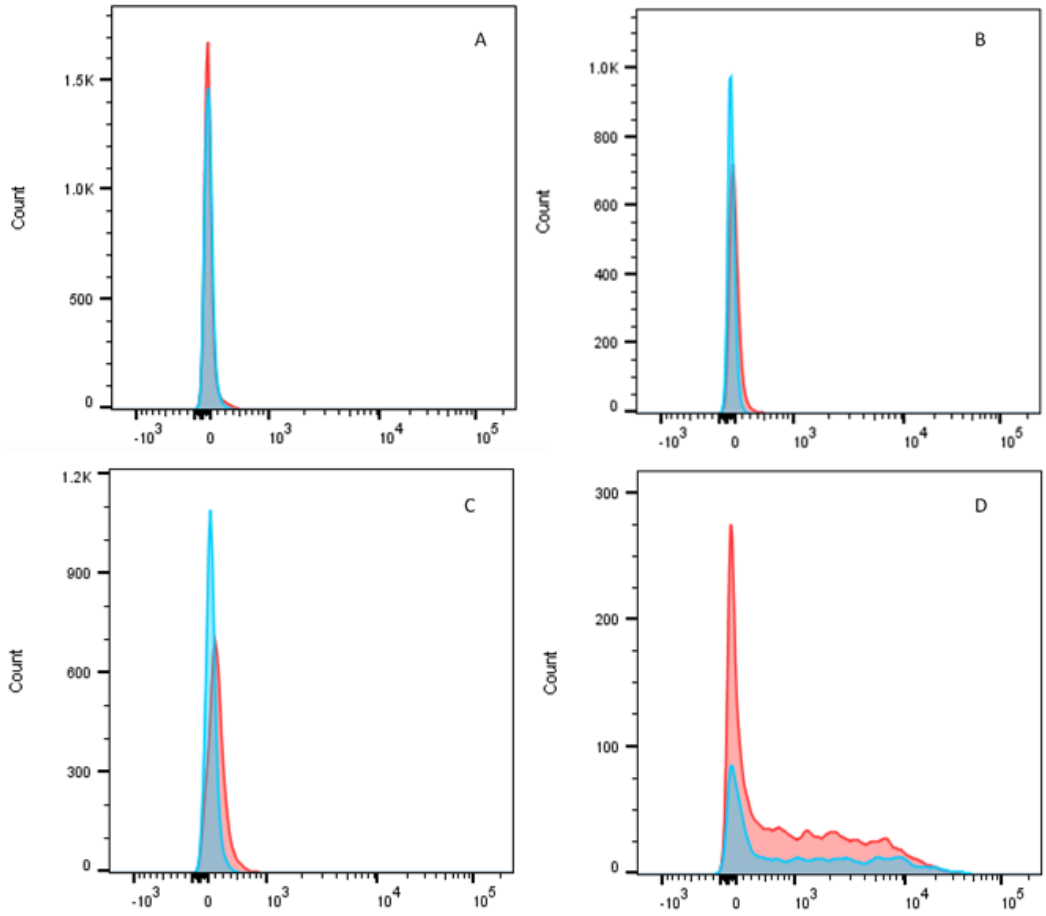
By Day 6 the high SSc granulocyte population observed at day 0 was completely lost and the the total percentage of cells expressing CSF-1, CD14, CD172a and CD16 plateaued or slightly increased as the maturing monocyte-macrophage cells began to acquire these surface proteins. There was a concurrent increase in the percentage of cells expressed ADGRE-1, and CD163.

By Day 10 all cells were adherent, larger (increased FSc), and more granular (increased SSc) than any of the cells present at Day 0 (as shown in Figure 11.4) and there is expression of CSF-1, CD14, CD172a, ADGRE-1 and CD163 as would be predicted for a pure population of mature macrophages (Figure 11.5). For each dog there was a variable proportion of cells observed at Day 10 which expressed CD16 (48-62%); suggesting that in dogs the divisions of classical, intermediate and non-classical monocyte subsets may exist, as has been demonstrated in human bone marrow though further functional assays are needed to confirm this (Mandl *et al.* 2014; van Leeuwen-Kerkhoff *et al.* 2018).

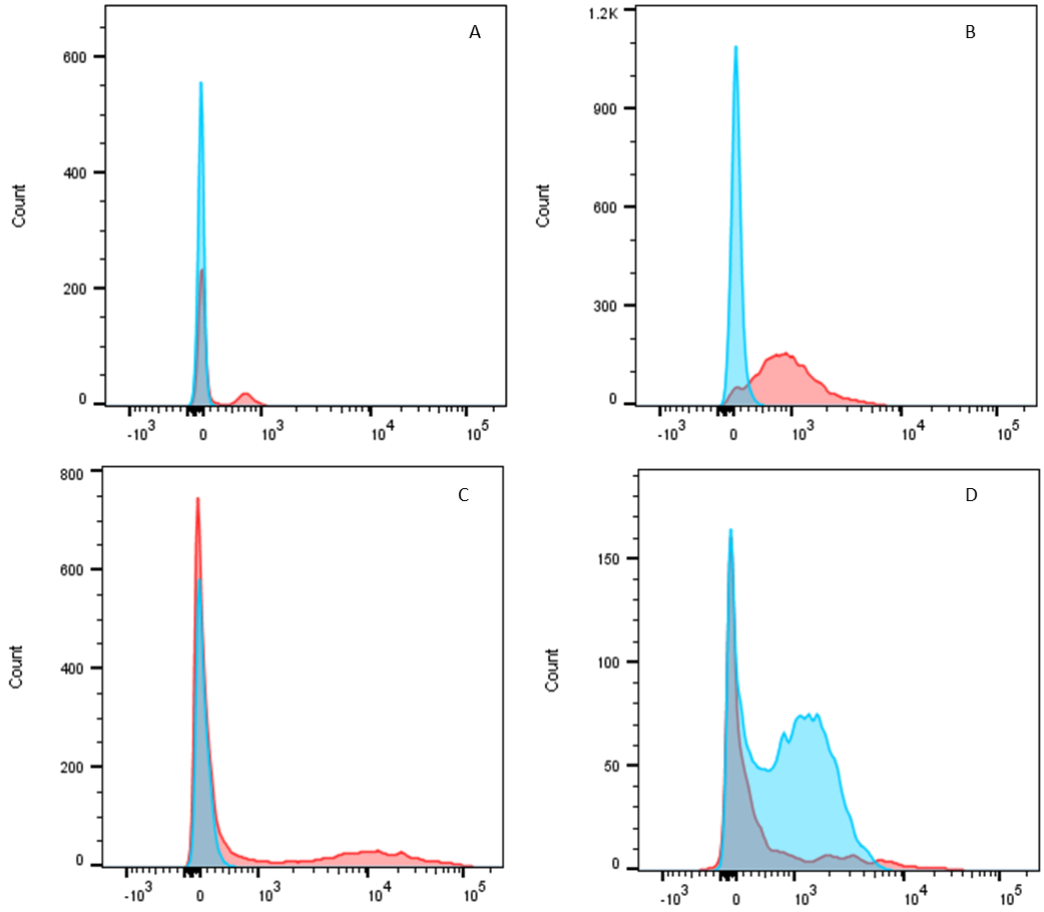
1: Surface CSF-1 staining



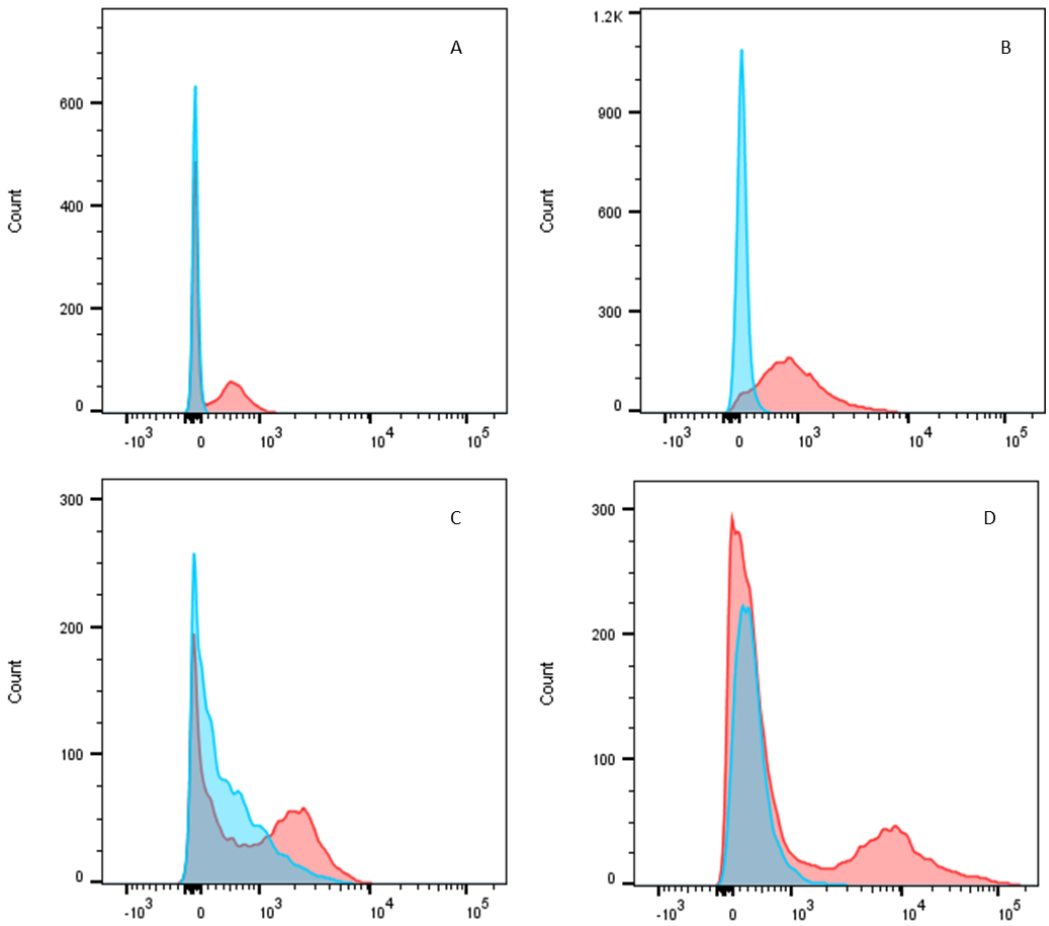
2: Surface ADGRE-1 staining



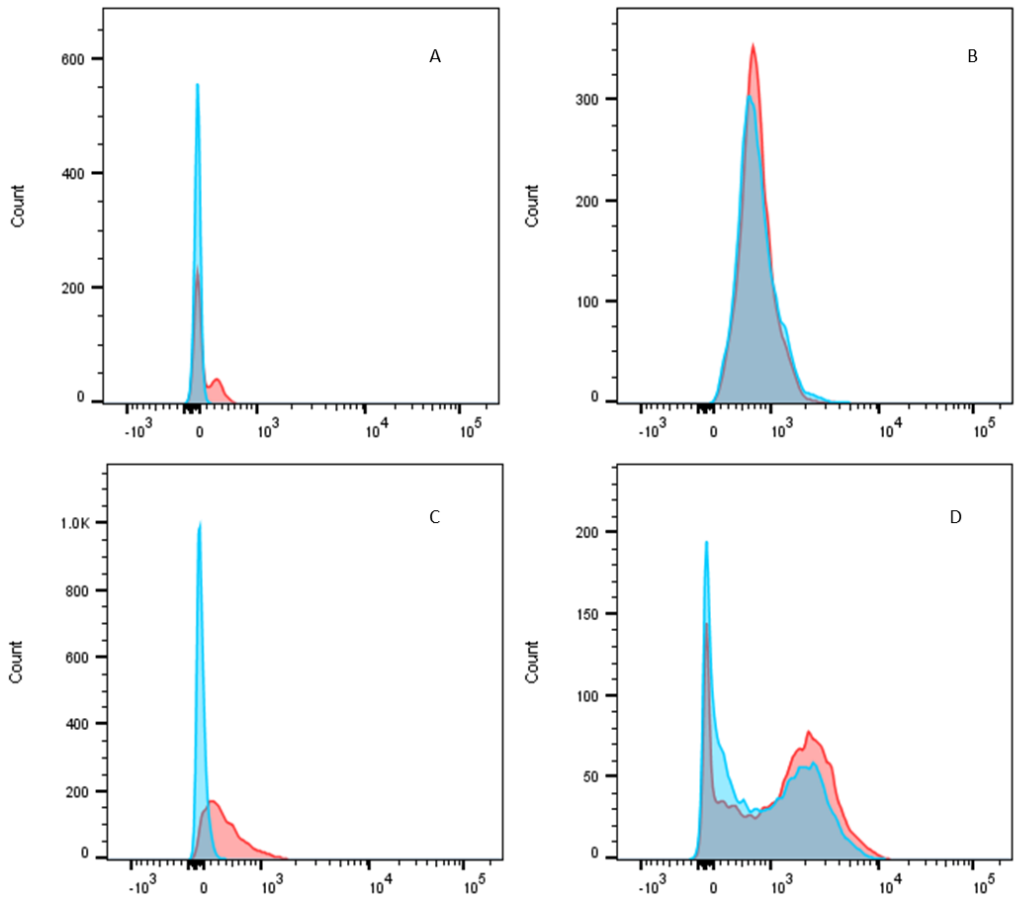
3: Surface CD172a staining



4: Surface CD163 staining



5: Surface CD16 staining



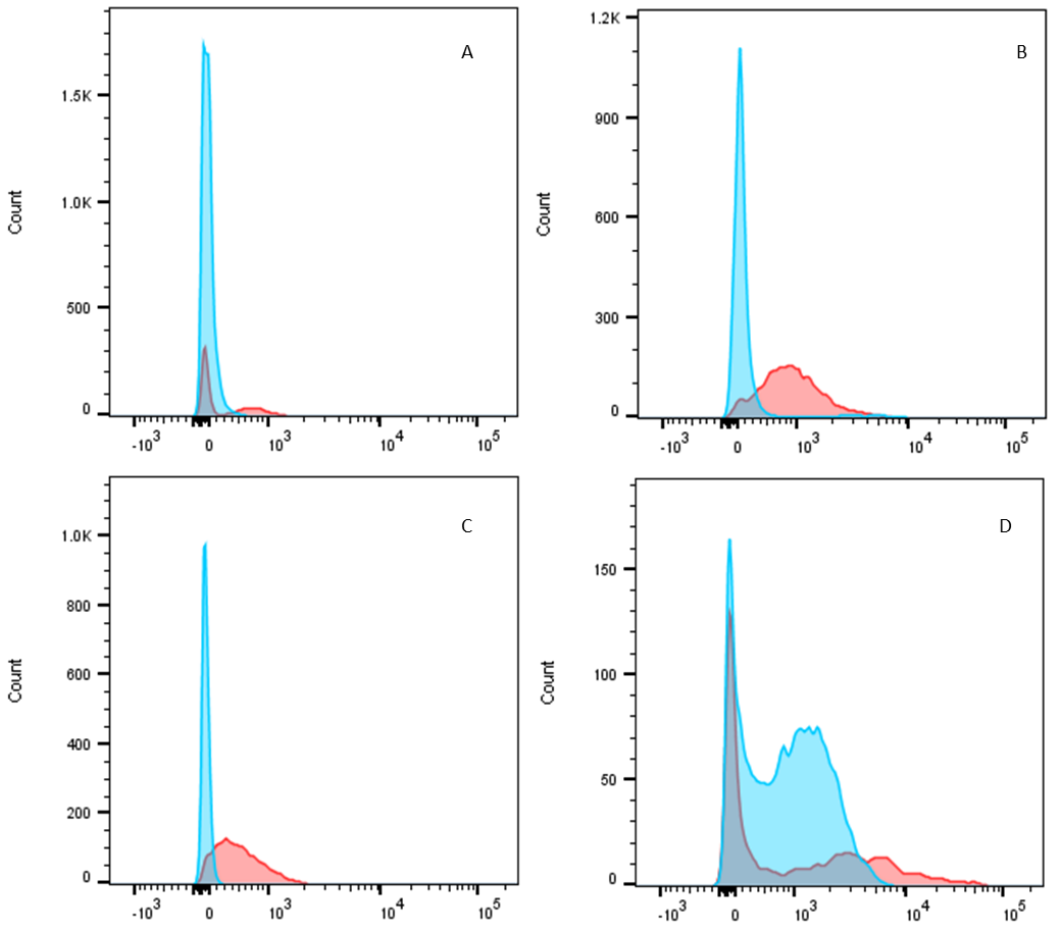


Figure 11.5: Histogram plots showing the changes in cell surface marker expression of canine bone marrow cells after 0 days (A), 3 days (B), 6 days (C) and 10 days (D) in r-CSF-1. Sets of plots are shown for each of surface CSF-1 (1), ADGRE-1 (2), CD172a (3), CD163 (4), CD16 (5) and CD14 (6). Stained cell populations are shown in red and isotype controls are shown in blue.

11.3.3 Both recombinant porcine and recombinant canine CSF-1 can be used to derive macrophages from canine bone marrow cells, equivalent to rh-CSF-1

Although rh-CSF-1 has been used previously to generate mature macrophages from peripherally circulating canine monocytes (Gow *et al.* 2013), since this finding was published a recombinant porcine (rp)-CSF-1 had become commercially available and a recombinant canine (rc)-CSF-1 was under development. The aim of this study was to assess the response of canine bone marrow cells to rp-CSF-1 and rc-CSF-1 and therefore establish if these growth

factors can maintain viable cells and drive macrophage differentiation equivalently or more efficiently than rh-CSF-1.

The biological activity of each r-CSF-1 was assessed by MTT assay. Bone marrow from eight dogs was cultured in either rp-CSF-1 (dogs 1-4) or rc-CSF-1 (dogs 5-8) with rh-CSF-1 at 10^4IU/mL included for all dogs as a positive control condition. Alongside this cells of the DH82 cell line, whose growth is CSF-1 independent were included cultured with rp-CSF-1. After ten days in culture the MTT assay was performed, the OD_{570} values were measured and the mean of three technical replicates is shown in Table 11.6.

Table 11.6: Mean OD_{570} of three technical replicates for each dog after ten days of culturing bone marrow in r-CSF-1. Dog 1-4 and DH82 cells were cultured in rp-CSF-1. Dog 5-8 cells were cultured in rc-CSF-1.

Concentration of CSF-1 (ng/mL)	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6	Dog 7	Dog 8	DH82
1000	0.35	0.36	0.01	1.49	0.13	1.64	1.09	2.10	2.55
500	0.25	0.23	0.01	1.47	0.11	1.23	0.71	0.78	2.54
250	0.12	0.17	0.01	1.06	0.06	1.01	0.66	0.23	2.53
125	0.05	0.14	0.01	0.65	0.05	0.79	0.52	0.08	2.48
62.5	0.05	0.13	0.01	0.55	0.04	0.56	0.48	0.04	2.50
31.25	0.05	0.08	0.01	0.04	0.011	0.64	0.35	0.02	2.50
10^4IU/ml rh-CSF-1	**0.32**	**0.34**	**0.01**	**1.38**	**0.08**	**0.65**	**0.54**	**0.18**	**2.51**

The OD_{570} values were plotted for each dog and joined by a curve of best fit (as shown in Figure 11.5). The equation of the line allowed the calculation of the concentration of rp-CSF-1 or rc-CSF-1 which had equivalent biological activity to 10^4IU/mL of rh-CSF-1, shown in Table 11.7.

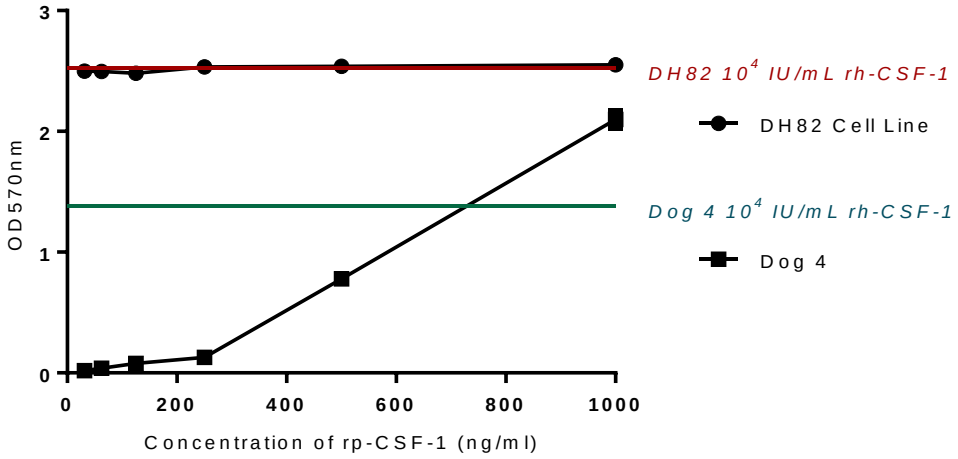


Figure 11.6: MTT assay for BMDM obtained from Dog 4 (■) and DH82 cells (●) cultured in varying concentrations of rp-CSF-1. A standard condition 10^4IU/mL rh-CSF-1 was used as a control for each cell type; shown by the red line for DH82 cells and the green line for Dog 4 BMDM. Dog 4 was one of four dogs used to evaluate the biological efficacy of rp-CSF-1.

Table 11.7: Concentration of recombinant (porcine/canine) CSF-1 (ng/ml) equivalent in biological activity to 10^4IU/ml rh-CSF-1 for each dog tested.

	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6	Dog 7	Dog 8	DH82
Concentration of rc/rp-CSF-1 (ng/mL) equal to 10^4IU/ml rh-CSF-1	887	905	∞	723	487	36	134	226	∞

While the MTT assays were used to establish cell viability in each r-CSF-1, the morphology of the cells generated was assessed after ten days in culture to ensure that the cells present were comparable to those generated by culture with rh-CSF-1. Both tested recombinant proteins generated cells with macrophage-like appearance as shown by representative images of the cells in culture (Figure 11.6).

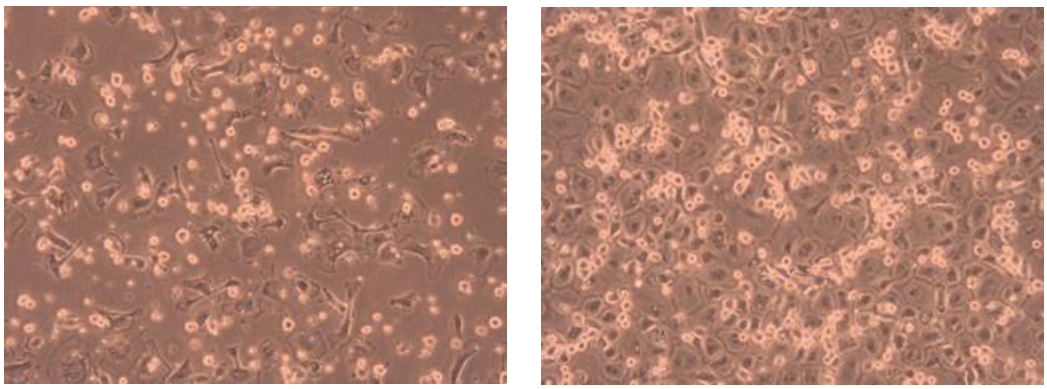


Figure 11.7: Representative images of cells in culture after ten days of differentiation in complete culture media supplemented with either rc-CSF-1 (Dog 4, left) or rp-CSF-1 (Dog 8, right).

All three recombinant proteins tested induced macrophage differentiation as determined by cell morphology and adherence to bacteriological plastic. As might be predicted, the canine protein was effective at lower final concentrations than the porcine protein; mean of 221ng/mL compared to 838ng/mL respectively. However, there was notable variation between the dogs tested. Bone marrow cells from one dog, Dog 3, did not respond at all to the recombinant porcine protein despite responding to rh-CSF-1 previously. The concentration of rc-CSF-1 equivalent to 10^4IU/mL of rh-CSF-1 ranged from 36-487ng/mL whilst the results for rp-CSF-1 ranged from 723-905ng/mL. Based on these findings the recombinant proteins were used at concentrations of; 10^4IU/mL rh-CSF-1, 850ng/mL rp-CSF-1 or 250ng/mL rc-CSF-1 to generate canine BMDM for further experiments.

11.3.4 Canine bone marrow dervided macrophages are phagocytic

A key aspect of macrophage function is their phagocytic capacity. To establish the phagocytic ability of the canine BMDM generated herein, differentiated cells from five dogs were assessed for their capacity to phagocytose zymosan particles and the resulting images are shown in Figure 11.7.

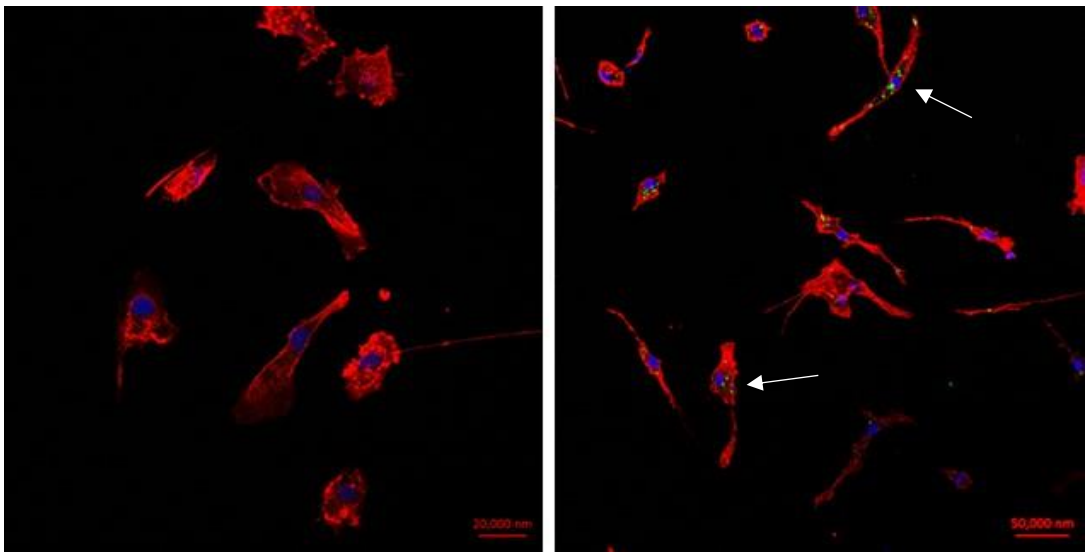


Figure 11.8: Canine BMDM differentiated from Dog 4 in rh-CSF-1 exposed to either PBS control (left) or FITC fluorescent Zymozan particles (right) showing uptake of particles (green, indicated by white arrows). Cellular filamentous actin was stained with Texas Red phalloidin (red) and nuclear material was counterstained with DAPI (blue). Imaged using a Zeiss LSM710 fluorescent microscope. One representative image of duplicate experiments from three dogs.

11.3.5 Canine BMDM are permissive to *Mycobacterium bovis* infection and demonstrate mycobactericidal activity

The main objective of developing a methodology for generating canine BMDM herein was to establish a model for the study of canine mycobacterial infections, and *Mycobacterium (M.) bovis* infections in particular. Previous studies that have examined the interaction of *M. tuberculosis* with human monocyte-derived macrophages, or *M. bovis* with bovine macrophages (Jensen *et al.* 2018) have utilised a multiplicity of infection of five (MOI=5). It was therefore necessary to establish whether this MOI was suitable for the study of canine BMDM-*M. bovis* interactions and to assess the number of mycobacteria required to achieve this in canine BMBM (Nazari *et al.* 2014; Ruiz *et al.* 2019).

The reference strain of *M. bovis* AF-2122/97 was cultured and quantified whilst BMDM were derived from a single dog in rc-CSF-1 (Dog 3). The *M. bovis* was diluted in complete culture media to a range of concentrations/estimated MOI and was then used to challenge $1x10^6$ BMBM/well *in vitro* as described in Section 11.2.8. Briefly, at the timepoints 0, 0.5, 1, 2 and 24

hours post infection the mycobacteria containing media was removed and cells washed thoroughly to allow the remaining macrophages to be lysed and plated to determine the number of viable bacteria that had been internalised. The number of CFU of *M. bovis* in these lysates was quantifed by culture and the MOI was retrospecitively calcultated. The results are shown in Table 11.8.

Table 11.8: The multiplicity of infection (MOI) calculated following different durations of challenge of canine BMDM with various concentrations of *M. bovis* AF-2122/97, n=1.

Concentration of *M. bovis*/mL of culture media	**Calculated MOI at 0.5 hours post challenge**	**Calculated MOI at 1 hour post challenge**	**Calculated MOI at 2 hours post challenge**	**Calculated MOI at 24 hours post challenge**
0	0.00	0.00	0.00	0.00
$1x10^6$	0.97	0.75	0.39	0.33
$2x10^6$	1.95	1.87	0.97	0.43
$5x10^6$	5.03	3.74	1.78	1.57
$1x10^7$	8.82	6.11	3.70	2.86

These data demonstrate that a MOI=5 was best achieved when cells were challenged with five *M. bovis* per macrophage for 30 minutes before the media was changed. This allowed mycobacteria to enter or be phagocytosed by BMDM, but was not long enough for significant macrophage mediated killing to occur. The data also demonstrate a notable, rapid decline in the recoverable CFU of *M. bovis* from the BMBM of this animal within a 24 hour period indicating potentially potent mycobactericidal activity.

To confirm this finding, the *in vitro* challenge was repeated with BMDM derived from six additional animals (Dogs 6-11, Table 11.9) and the CFU of *M. bovis* recovered at each of four time points (0, 2, 6 and 24 hours post infection; Figure 11.8). Media containing *M. bovis* containing media was added to canine BMDM and left for 30 minutes before being removed, the cells were then washed to remove any extracellular bacilli and complete culture media added. The time point ‘0 hours post infection’ was defined as the time that complete culture media without *M. bovis* was added to the tissue culture wells.

Table 11.9: Signalment details of the dogs from which canine BMDM cells were derived and infected with *M. bovis*.

Dog Number	Breed	Age (years)	Sex
Dog 6	Beagle	Three	Male Entire
Dog 7	Beagle	Three	Male Entire
Dog 8	Beagle	Four	Male Entire
Dog 9	Beagle	Three	Male Entire
Dog 10	Beagle	Four	Male Entire
Dog 11	Beagle	Four	Male Entire

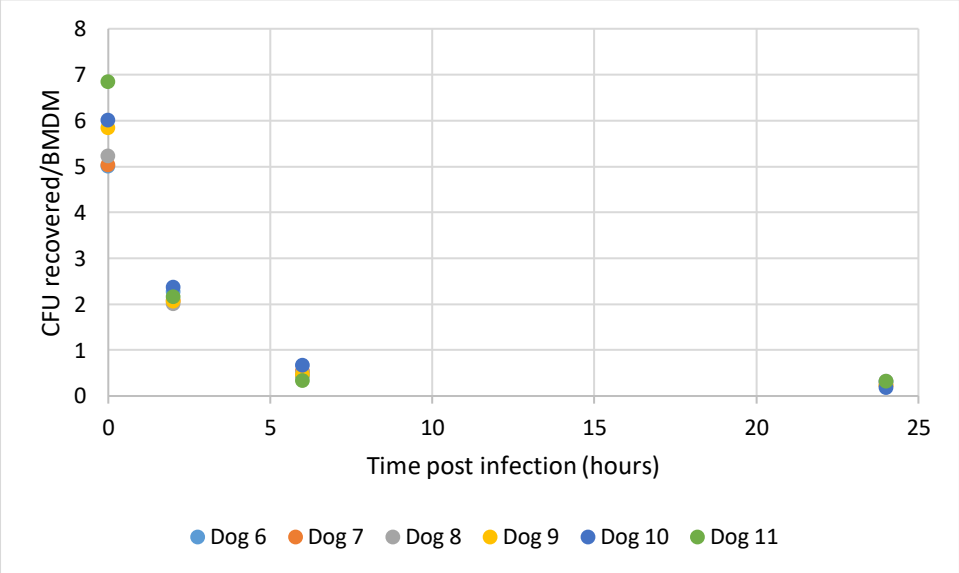


Figure 11.9: Canine BMDM from six dogs were grown for ten days in rh-CSF-1 and exposed to *M. bovis* at a predicted MOI of 5. At 0.5h post-infection cells were washed and media replaced. The number of CFU was calculated at the indicated time points post-infection in lysates from infected cells. Data represent the mean of duplicate CFU counts.

This *in vitro* infection experiment confirmed the initial finding that canine BMDM display mycobacericidal activity; within the first six hours post infection there is a ten-fold reduction in the recoverable CFU count from approximately 5 mycobacteria/BMDM cell to 0.5 mycobacteria/BMDM cell. The rate of killing then slows considerably and by 24 hours post infection the CFU count halves to approximately 0.25/BMDM.

11.3.6 Canine BMDM and DH82 cells respond to lipopolysaccharide stimulation and mycobacterial infection by producing pro-inflammatory cytokines but not nitrite

Based on the key findings of studies in other species, the study presented here examined the proinflammatory response of canine BMDM and the DH82 cell line by quantifying the proinflammatory cytokines IL-6 and TNF-α in response *Salmonella enterica* serotype Minnesota strain 595 derived LPS, the attenuated *M. bovis*-BCG and virulent *M. bovis.* The BMDM were derived from three dogs (Dog 6, Dog 7 and Dog 8) and DH82 were routinely cultured. Cells were either stimulated with LPS or infected with mycobacteria. Supernatants from unstimulated control cells and the challenged cells were taken at 0 hours, 1 hours, 2, hour, 4 hours, 7 hours and 24 hours post infection. The supernatants were stored at -80°C before subsequent analysis by ELISA for the presence of IL-6 and TNF-α by ELISA and by Griess assay to determine the concentration of nitrite present. The cytokine response of the cells is shown in Figure 11.9; no nitrite was detected in any of the samples tested (data not shown).

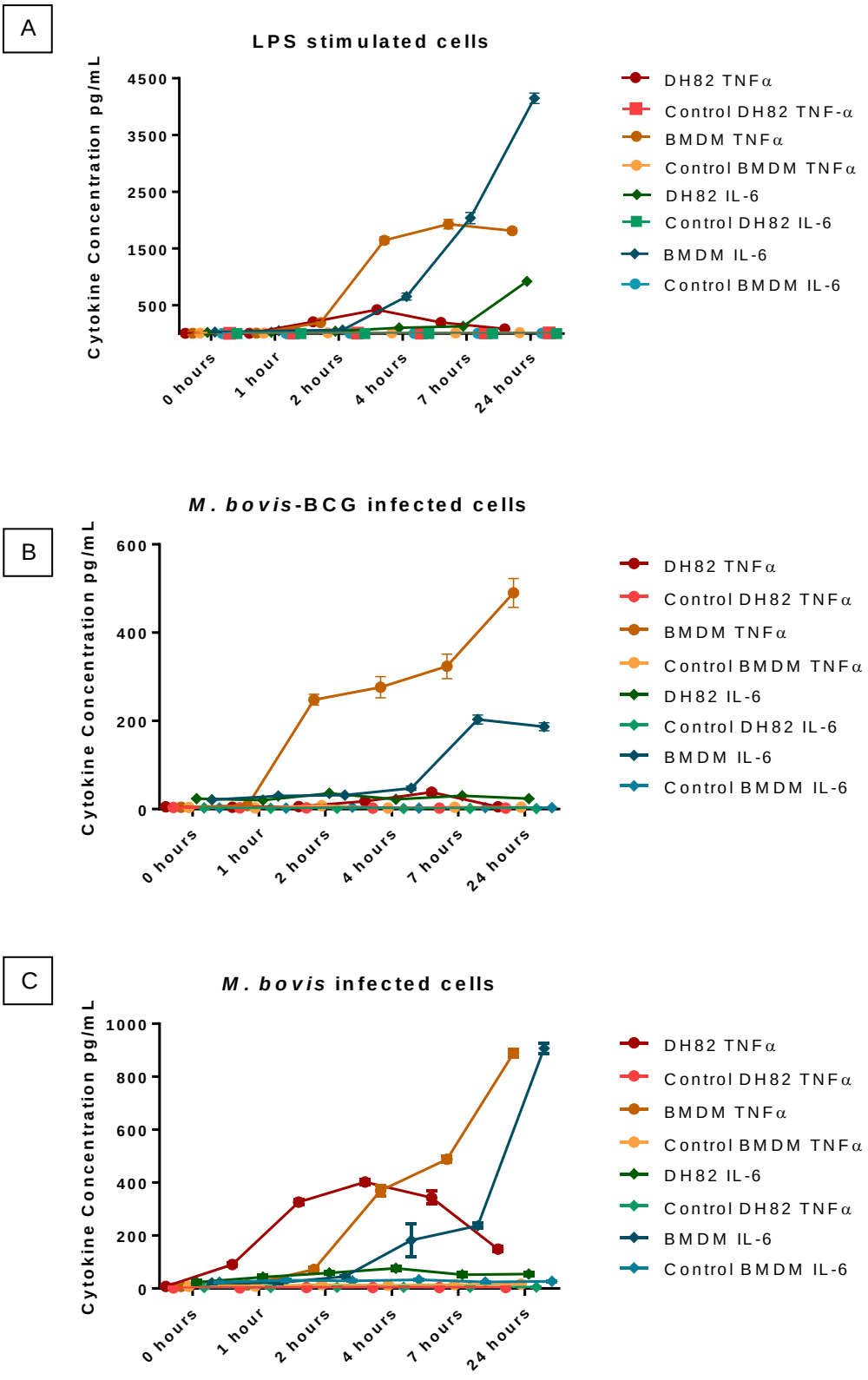


Figure 11.10: The TNFα and IL-6 responses of DH82 cells and canine BMDM to LPS stimulation (A), *M. bovis*-BCG infection (B) or *M. bovis* infection (C) compared to uninfected/unstimulated BMDM (control BMDM) and DH82 cells (control DH82). Data points are the mean of three biological and two technical replicates for BMDM and triplicate experiments for DH82 cells. Error bars show the standard deviation.

11.3.7 Canine BMDM and DH82 cells show different gene expression profiles in response to stimulation with LPS and infection with mycobacteria

Due to the limitations of available immunological reagents for domestic dogs, futher comparison of the canine macrophage response to LPS, *M. bovis*-BCG and virulent *M. bovis* required the development of a RT-qPCR protocol to examine gene expression rather than to detect protein production and secretion. Therefore, it was first necessary to identify genes in canine BMDM and the DH82 cell line which retained stable expression levels under all three challenge conditions for use as reference genes.

An initial panel of six genes was selected as shown in Table 11.2. These primer combinabtions were assessed by perfroming RT-qPCR on reverse transcriptase positive and negative samples. The RNA samples tested were collected from each timepoint and for all three stimuli. The primers that amplify hypoxanthine phosphoribosyltransferase 1 (HPRT-1, primer pair one), ribosomal protein S7 (RPS7, primer pair one), ribosomal protein L-23 (RPL-23, primer pair two) were the only three to give a single RT-qPCR product as determined by melt curve analysis (shown in Figure 11.12), and gave a stable expression level as assessed by the cycle threshold (Ct) values across all conditions and with both cell types, shown in Table 11.10.

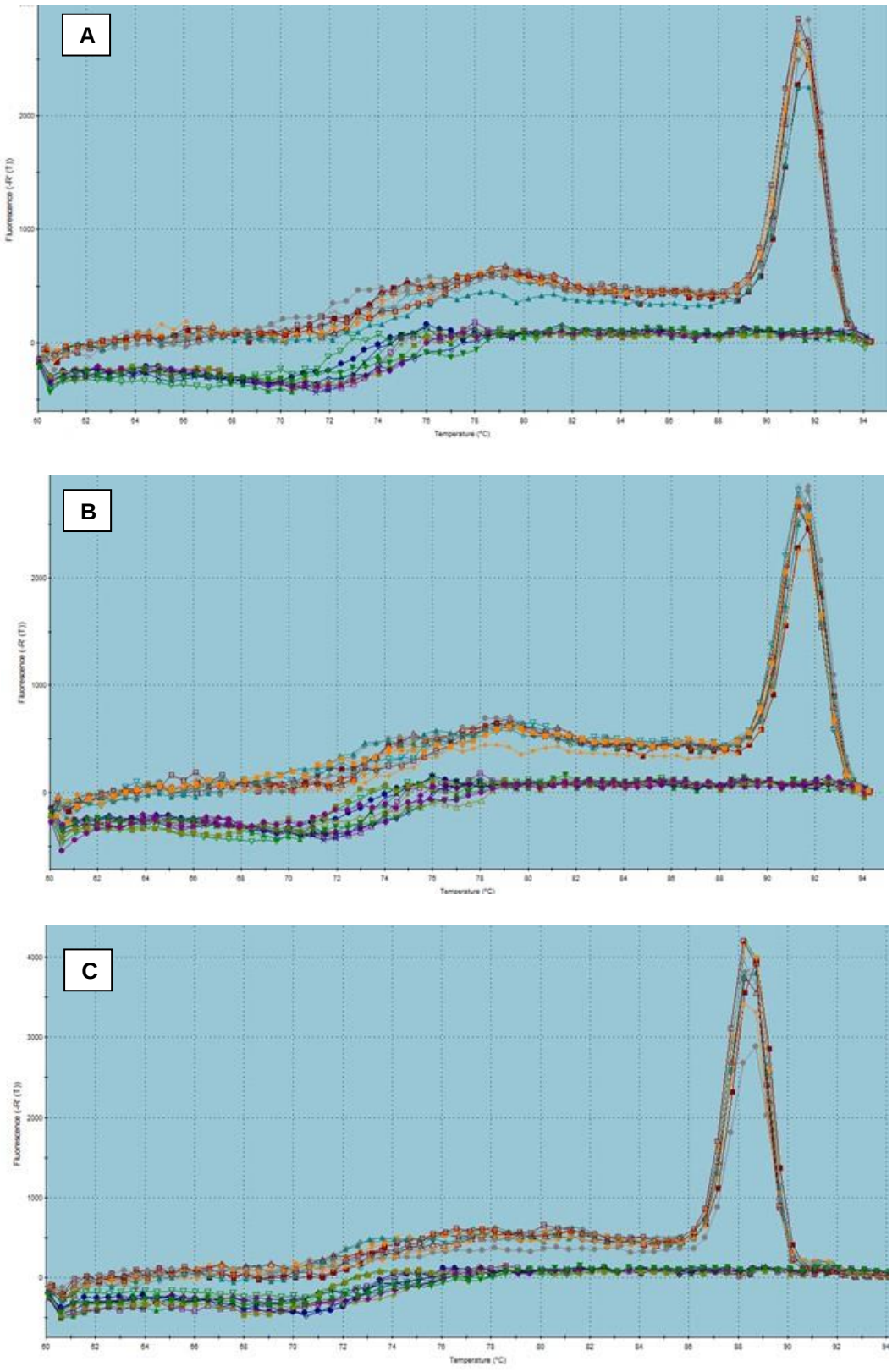


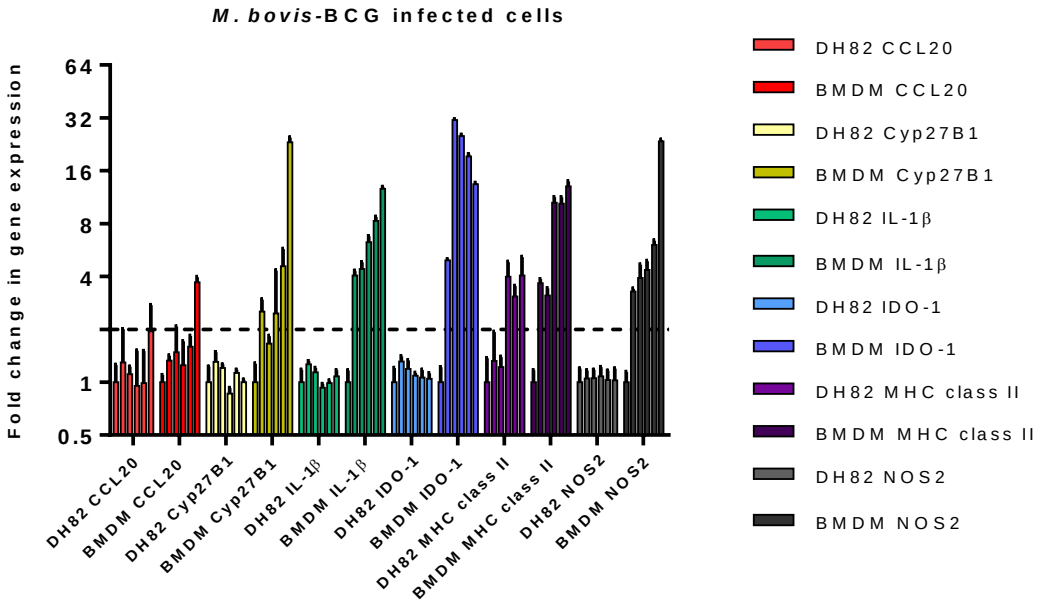
Figure 11.11: Dissociation curves for the three primer pairs, HPRT-1 [A], RPS-7 [B] and RPL-23 [C] that produced single RT-qPCR products and stable Ct values when tested.

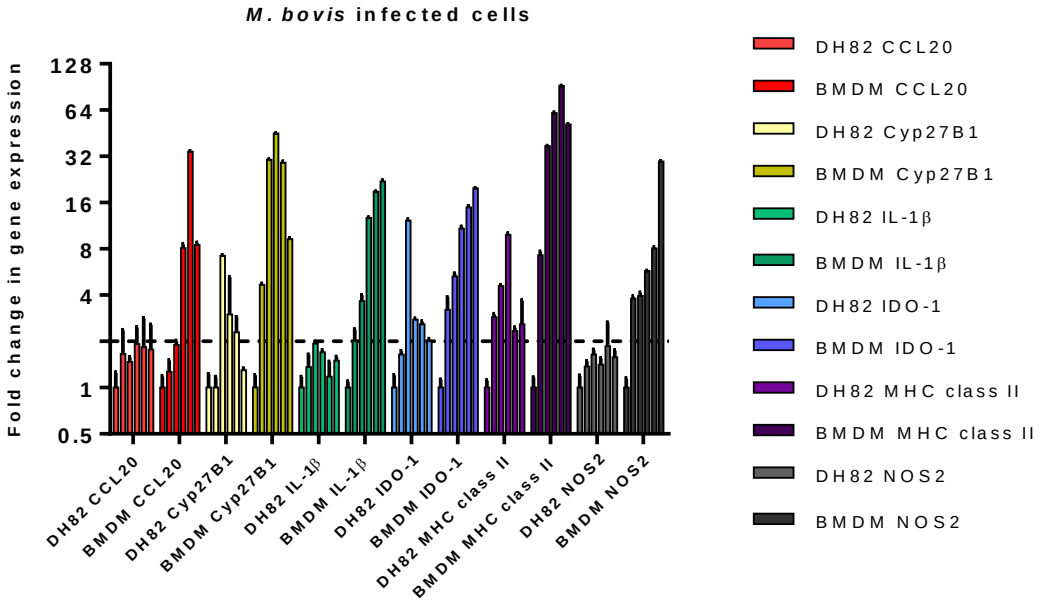
Table 11.10: The mean of duplicate Ct values for each candidate reference gene analysed at each timepoint, for both cell types and with all three stimuli.

Primer Pair	Cell Type	Stimulus	0 hours	1 hour	2 hours	4 hours	7 hours	24 hours
HPRT-1	DH82	*M. bovis*	25.35	25.22	24.81	25.33	25.58	25.04
HPRT-1	DH82	*M. bovis* BCG	25.15	25.14	25.32	25.18	24.79	24.66
HPRT-1	DH82	LPS	25.12	25.05	25.21	25.73	25.43	25.28
HPRT-1	BMDM	*M. bovis*	24.75	25.14	25.32	25.78	24.79	24.66
HPRT-1	BMDM	*M. bovis* BCG	25.12	25.05	25.21	25.73	25.43	25.28
HPRT-1	BMDM	LPS	25.13	25.36	24.40	25.57	25.59	25.55
RPS7	DH82	*M. bovis*	26.12	26.2	26.23	25.9	26.16	25.98
RPS7	DH82	*M. bovis* BCG	26.18	26.08	26.42	25.97	26.13	25.85
RPS7	DH82	LPS	26.74	25.96	26.31	25.99	25.89	26.15
RPS7	BMDM	*M. bovis*	26.97	25.84	26.40	26.04	25.79	26.16
RPS7	BMDM	*M. bovis* BCG	27.28	25.72	26.44	26.09	25.66	26.25
RPS7	BMDM	LPS	27.19	25.60	26.48	26.13	25.52	26.33
RPL-23	DH82	*M. bovis*	31.15	31.06	30.83	31.15	30.91	30.40
RPL-23	DH82	*M. bovis* BCG	30.83	30.91	30.93	31.18	31.21	31.14
RPL-23	DH82	LPS	31.21	30.82	31.09	31.15	30.93	30.40
RPL-23	BMDM	DH82	30.94	30.96	31.29	30.92	30.77	30.86
RPL-23	BMDM	*M. bovis*	30.97	30.84	31.42	30.92	30.78	30.86
RPL-23	BMDM	LPS	30.95	30.80	31.57	30.85	30.71	30.93

In murine macrophages, nitrite is measurable in the cell supernatant following stimulation with LPS due to the upregulation of inducible nitric oxide synthase 2 (NOS2) and the metabolism of arginine to produce nitric oxide, an unstable free radical which rapidly forms nitrite in solution (Kapetanovic *et al.* 2012). In porcine BMDM, this process does not occur and neither the nitrite product nor NOS2 gene transcript are found following exposure to LPS and instead, pig BMDM alternatively upregulate L-tryptophan metabolism via indoleamine 2,3-dioxygenase 1 (IDO1) (Kapetanovic *et al.* 2012). To confirm the lack of nitrite production that suggests both DH82 cells and primary canine BMDM respond similarly to pig BMDM, the expression of NOS2 and

IDO1 were quantified in both cell types following challenge with each of LPS, *M. bovis*-BCG and *M. bovis* as per Section 11.3.4. The gene expression of major histocompatibility complex II (MHC class II), IL-1β, the lymphoattractant chemokine CCL20 (also called MIP3α) and the vitamin D3-converting enzyme, Cyp27B1 were also measured by RT-qPCR in the same samples, normalised to HPRT-1 and RPS7. The results are given as a fold-change in gene expression compared to resting cells (*i.e.* time 0 hours post infection). Expression changes of greater than 2-fold from resting were considered to be biologically significant and are shown in Figure 11.11.


M. bovis-BCG infected cells
Fold change in gene expression
64
32
16
8
4
1
0.5
DH82 CCL20
BMDM CCL20
DH82 Cyp27B1
BMDM Cyp27B1
DH82 IL-1β
BMDM IL-1β
DH82 IDO-1
BMDM IDO-1
DH82 MHC class II
BMDM MHC class II
DH82 NOS2
BMDM NOS2


M. bovis infected cells
Fold change in gene expression
128
64
32
16
8
4
1
0.5
DH82 CCL20
BMDM CCL20
DH82 Cyp27B1
BMDM Cyp27B1
DH82 IL-1β
BMDM IL-1β
DH82 IDO-1
BMDM IDO-1
DH82 MHC class II
BMDM MHC class II
DH82 NOS2
BMDM NOS2

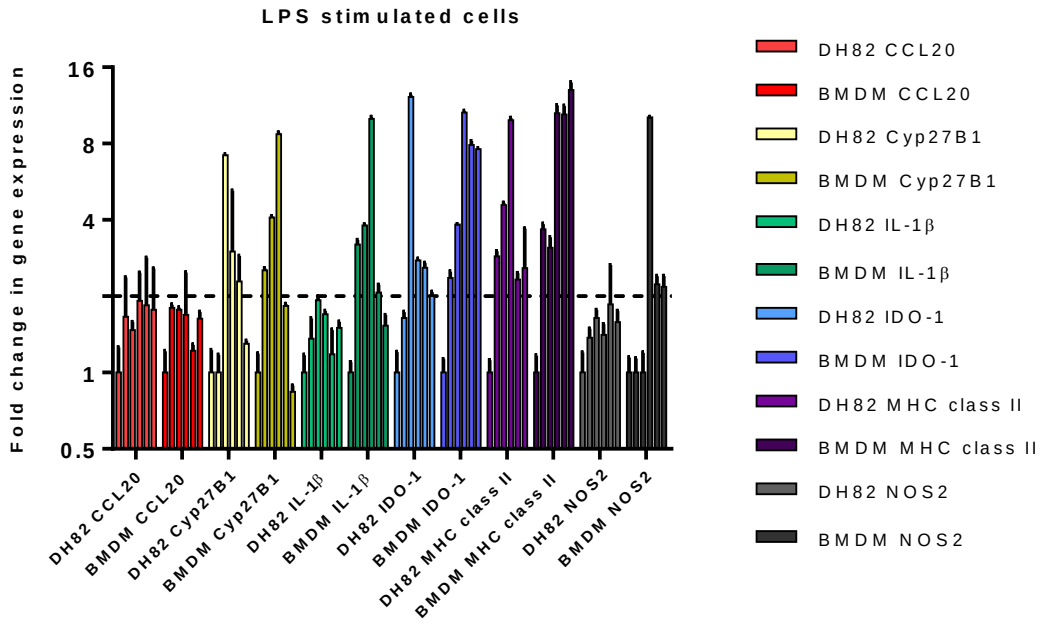


Figure 11.12: The fold-change of gene expression after stimulation with LPS or infection with *M. bovis*-BCG or *M. bovis* for both DH82 and canine BMDM cells. Gene expression was compared to resting state for the six genes of interest, normalised to averaged HPRT-1 and RPS7 expression. The six bars represent the timecourse analysed; sequentially left to right showing 0 hours, 1 hours, 2 hours, 4 hours, 7 hours and 24 hours post infection/stimulation. Bars show the mean of three biological (Dog 6, 7 and 8) and two technical replicates for BMDM, and two replicate experiments for DH82 cells. Error bars show the standard deviation. The dashed line represents a 2-fold change which is considered biologically significant.

The data presented in Figures 11.9 and 11.11 together demonstrate that canine BMDM display archetypal pro-inflammatory responses to all three stimuli examined; LPS, *M. bovis*-BCG and virulent *M. bovis.* The cytokines IL-6 and TNF-α were both secreted at detectable levels by four hours following stimulation. Post-stimulation, canine BMDM also significantly increased their expression of both MHC class II and of IL-β. Together these show conclusively that the BMDM generated herein are capable of adopting the classically activated phenotype. By comparison, the frequently studied DH82 cells also produced TNFα and IL-6 and significantly upregulated MHC class II when stimulated but did not show any change in IL-1β mRNA levels. There were also other differences between the two cell types in their response to the three stimuli examined.

In general, the BMDM responded more potently to all stimuli compared to DH82 cells. This difference was most clearly seen in response to *M. bovis*-BCG infection, which had a nearly negligible effect on DH82 cells over 24 hours with only small concentrations of IL-6 and TNF-α secreted and only MHC class II was upregulated across the panel of six genes studied. In contrast, BMDM upregulated Cyp27B1, IL-1β, IDO-1, MHC class II and NOS2 gene expression as well as secreting significant amounts of both TNF-α (from two hours post infection) and IL-6 (from seven hours post infection). In response to *M. bovis* the concentration of TNF-α secreted by DH82 into the supernantant peaked at approximately 400pg/mL at four hours post infection before declining, whereas the concentration produced by BMDM continued to rise across the 24 hours time course, peaking at approximately 800pg/mL. The DH82 cells failed to induce IL-1β gene transcription under any of the conditions studies, which is supported by previous studies showing that the protein is not secreted when the cells are infected with intracellular parasites such as *Leishmania* spp (Maia *et al.* 2007).

11.4 Discussion

The aim of the study presented here was to utilise recombinant CSF-1 to generate canine macrophages from bone marrow in order to chararcterise them and then to examine the effect of TLR agonism and, critically, to investigate the early response of these cells to mycobacterial infection. An additional goal was to compare the response of the generated canine BMDM to the selected stimuli with the response of the frequently used DH82 cell line in order to evaluate the usefulness of both systems as models for studying canine mycobacterial infections.

The data presented here demonstrate that the methodology developed herein can be used to generate macrophages from canine bone marrow. In keeping with BMDM from humans and pigs, the cells were found to be adherent, have typical macrophage morphology, and phagocytic capacity; all hallmark features of mature cells of the mononuclear phagocyte system. This study is the first to use bone marrow from dogs to generate macrophages where previous studies have instead used circulating monocytes. The major advantage of bone marrow as a starting source of cells is the ability to obtain many more cells from a single animal; allowing multiple experiments to be conducted with cells from a consistent background in terms of age, sex and breed that may all influence mature macrophage biology. This advantage is further conferred by the methodology shown here to cryopreserve bone marrow cells and still derive macrophages on recovery, meaning that significantly fewer animals may be needed for studies.

Established culture protocols for the generation of canine monocyte derived macrophages requires seven days to produce mature cells even though the initial cell population is commited to the lineage (Heinrich *et al.* 2017). By comparison, the protocol produced here for BMDM takes ten days, only three days longer, suggesting that the use of r-CSF-1 in the method described in this study may act to drive bone marrow progenitors to commit to a macrophage fate and drives their maturation giving rise to robust differentiation protocol. The use of r-CSF-1 also adds confidence that the final cell population is comprised purely of macrophages as

their survival is maintained *in vivo* by the action of CSF-1. This study is the first time that the surface expression of CSF-1 has been demonstrated on canine macrophages (Hume, 2018).

Although the protocol almost always resulted in the generation of canine BMDM, individual animals varied quite considerably in their responsiveness to r-CSF-1, particualy when different r-CSF-1 molecules were directly compared. As this study only included a relatively small number of animals it was not possible to identify any individual reason for this variation in response; however, it may be a combination of factors such as breed, age and sex of the dog that the bone marrow cells were obtained from. Studies with a more homogenous population of dogs would be useful to gain better insight into the effect of these factors on the differentiation potential of bone marrow cells and their responsiveness to CSF-1 as this may also contribute to the susceptibility of an individual to infection and/or disease from mycobacterial challenge.

Previous characterisation of canine monocyte derived macrophages has shown that they express the lineage associated surface molecules CD14 and CD163 whilst a subset express CD16 (Heinrich *et al.* 2017). Examination of histiocyte cells lines, such as DH82 cells, has additionally shown expression of CD172a (Takada *et al.* 2018). The data presented here show that the canine BMDM produced display the same expression pattern of these cell surface molecules; mature cells were consistently (over 95%) positive for CD14, CD163 and CD172a whilst just more than half expressed CD16 though this varied slightly from dog to dog. Numerous studies have divided blood monocytes from many species into subsets according to their expression of the surface markers CD14 and CD16; classical monocytes highly express CD14 but do not express CD16 (*i.e.* they are $CD14^{++}CD16^{-}$), intermediate cells also highly express CD14 but also express CD16 ($CD14^{++}CD16^{+}$) whilst non-classical monocytes express lower levels of CD14 and have high surface CD16 expression ($CD14^{+}CD16^{++}$) (Mandl *et al.* 2014; Ziegler-Heitbrock *et al.* 2014). In circulation, the classical phenotype dominates against lower numbers of the remaining two subtypes, but only one study has evaluated the presence of these subsets in the bone marrow of humans and has shown that the intermediate cell phenotype dominates (Mandl *et al.* 2014). In the present study, CD16 was co-expressed

with CD14 on both granuloctyes and monocytes (as defined by their forward and side scatter profiles) in undifferentiated bone marrow but this increased during the differentiation process so that by Day 10 a significant population of the macrophages were intermediate or non-classical in phenotype, which is the first time that this has been shown in dogs. In this study it was not possible to differentiate $CD14^{+}$ and $CD14^{++}$ cells at Day 10 due to all cells being beyond the visual field; this was done to keep the cytometer parameters identical at all time points but this could be altered in future studies. Similarly, different subsets of human BMDM cells have been shown to have different biological functionality such as altered chemokine receptor expression profiles (Mandl *et al.* 2014). Therefore future studies of canine BMDM could separate the cells into phenotypic subsets and further explore their individual response to stimuli or infection.

The F4/80 antigen, encoded by the *Adgre1* locus, has been widely-used as a monocyte-macrophage marker in mice, but its value as a macrophage marker in other species is unclear, and has even been questioned (Hume, 2018; Waddell *et al.* 2018). It has been previously demonstrated that ADGRE-1 is a myeloid differentiation marker in pigs, absent from progenitors in bone marrow, highly-expressed in mature granulocytes, monocytes, and tissue macrophages and induced by CSF-1 treatment *in vivo* (Hume, 2018; Waddell *et al.* 2018). Here, the same expression pattern of ADGRE-1 was demonstrated in canine macrophage progenitors in bone marrow and BMDM using the same antibody clone as the porcine study, which showed good cross-reactivity to dog. However, the granulocytes within the canine bone marrow did not stain positively for ADGRE-1 which was unexpected but was beyond the scope of this study to investigate further; future characterisation of the immunophenotype of cells within canine bone marrow would determine which cell types could be derived and studied *ex vivo*.

Similarly to the expression of ADGRE-1, canine BMDM may be similar to porcine BMDM in their pattern of producing TNF-α in response to LPS stimulation but not generating detectable nitrite. However, RT-qPCR analysis showed that the canine BMDM and DH82 cells did significantly upregulate NOS2 gene expression from four hours post LPS stimulation and so

extending the timecourse of this experiment may have revealed production at a later stage post stimulation. However, the production of mRNA does not necessarily always correlate with the induction of functional enzyme activity as a host of post-transcriptional modifications may occur which prevent translation. Alternatively, other reactive nitrogen species may be syntesised that are not detected by the Griess assay. The NOS2 enzyme acts to produce nitric oxide, an unstable radical that cannot be directly assayed and so nitirite is measured as a proxy. Canine BMDM may have redox mechanisms to safely deal with free radicals which may, in this case, produce alternative products to nitrite.

The genes for CCL20, Cyp27B1 and IDO-1 were examined by RT-qPCR analysis as they represent different aspects of the host response to tuberculosis infections. The chemokine CCL20 attracts lymphocytes and immature dendritic cells to the site of infection and is expressed by *M. tuberculosis* infected human monocyte derived macrophages (Rivero *et al.* 2010). The Cyp27B1 enzyme converts vitamin D3 which is a consistent feature of granulomatous disease processes, including tuberculosis (Chandra *et al.* 2004). Tryptophan metabolism is governed by IDO-1 and *M. tuberculosis* infection induces marked upregulation of IDO-1 expression in both human and murine macrophages *in vitro* (Blumenthal *et al.* 2012). In reponse to both mycobacterial infections (*M. bovis* and *M. bovis*-BCG) canine BMDM significantly (2-fold or greater) increased their expression of CCL20 at four, seven and 24 hours post infection while IDO-1 and Cyp27B1 mRNA was present at significant levels from one hour and then at each subsequent time point of the infection time course examined. These data suggest that similar to other species, these genes and the metabolic pathways they belong to, are important factors in mycobacterial immunity and that the canine BMDM model developed herein is a more biologically appropriate model for the study of canine mycobacterial infections than the DH82 cell line.

Further studies are needed to confirm and expand on the data shown here; further functional assessment of the cells such as quantification of phagocytic capacity and their ability to acidify phaogomes when stimulated/infected would add the the initial evidence that these cells are in fact macrophage. Future experiments could investigate the production of reactive nitrogen

species and nitrite by titrating concentrations of LPS and extending the timecourse of the challenge study which were logisitically not possible in this study but could be considered for future experiments.

In conclusion; the data presented here demonstrate that canine BMDM can be generated using r-CSF-1 supplemented culture media, during culture the resultant cells gain surface expression of key macrophage associated proteins. After ten days of differentiation, cells are a pure population of adherent cells which are morphologically macrophage-like and are phagocytic. Canine BMDM derived in r-CSF-1 comprise both $CD16^-$ (classical) and $CD16^+$ (intermidate/non-classical) subsets. These BMDM are permissive to mycobacterial infection and may represent a more biologically appropriate model for the study of canine tuberculosis infections than the commonly used DH82 cell line. These results could be expanded on by futher evaluation of the immunophenotypic subsets of canine BMDM and their potentially differing functionality as well as comparing different mycobacterial species and strains.

11.5 References

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Chapter 12: Final Discussion

In the UK, tuberculous mycobacterial infections were historically widely recognised in both cats and dogs (Anon, 1892; Anon, 1899). In the early 20th century the incidence of tuberculosis (TB) in the human population of the UK was as high as 300 cases per 100,000 of the population (Glaziou *et al.* 2018). Canine TB caused by the human adapted *Mycobacterium (M.) tuberculosis* first received significant attention in the UK in the late 1920s and early 1930s when it was shown that companion dogs can act as both spill-over hosts and as a sentinel species for human infections (Moore, 1928; Thayer *et al.* 1930; Adams and Vokwald *et al.* 1932; Steinbach and Deskowitz, 1933). Similarly, zoonotic TB (caused by *M. bovis* infection) was commonplace (Glaziou *et al.* 2018). Until the end of the Georgian era, most of a city's milk supply would come from cows living in sheds within city limits, increasing the risk of direct spread of TB, either by milk or airborne infection, to city dwellers (Wallace and Crouch, 1933; Tobiesen *et al.* 1934; Galbrieth *et al.* 1982). During the 1930's tuberculin testing was introduced for cattle in the UK; 40% of all cows were found to be reactors (Glaziou *et al.* 2018). With the introduction of pasteurisation, initially to prolong the shelf-life of milk, came the ability to control the major route of transmission of *M. bovis* disease to humans (Meara, 1976). At the same time, forty years of research had focussed on the dog as a model of primary pulmonary TB caused by both *M. bovis* and *M. tuberculosis,* whilst cases of feline TB due to *M. bovis* were regularly being recorded almost exclusively with alimentary pathology, presumptively following the ingestion of contaminated milk (Lemke, 1901; Lewis and Montgomery, 1913; Manwaring, 1913; Adams and Volkard, 1932; Howe, 1939a; Howe, 1939b). Interestingly, the apparent resistance of dogs to clinical disease and the lack of pathology in cats caused by *M. tuberculosis* had already been noted (Beretta, 1891; McFadyean, 1891).

With the advent of vaccination, the development of antimicrobial agents, improvements in socio-economic conditions and the formation of the National Health Service, between 1940 and 1990 the incidence of TB in the human population throughout the UK declined by 90% and the mortality rate fell by 98% (Glaziou *et al.* 2018). Throughout the same period, reports of companion animal TB in the literature saw a similarly dramatic decline and began to reflect

individual and sporadically occurring canine cases whilst reports of feline mycobacteriosis became largely limited to incidental findings during *post mortem* surveys (Dodd, 1952, Cordes *et al.* 1963; Birn *et al.* 1965).

Significant recognition of feline TB as a re-emerging clinical entity in the UK entered the veterinary literature again in the late 1990's with a description by Gunn-Moore *et al.* (1996) of 19 cases of disease due to "an unusual mycobacterial variant" which retrospectively was likely to be *M. microti* (the vole bacillus).

This major publication lead on to more in depth investigations into feline mycobacteriosis in the UK. Following the development of a feline interferon gamma release assay (IGRA) for cats in 2008 and with state funding made available for the gold standard test of specialist mycobacterial culture at the Weybridge reference laboratory, it was recognised that these infections were much more common than had previously been appreciated (Rhodes *et al.* 2008). In 2011, two seminal papers were published by Gunn-Moore *et al.* (2011a, 2011b) describing feline mycobacteriosis in extensive detail. A follow-up study, published in 2013 (Gunn-Moore *et al.* 2013), used histopathological submissions to estimate the incidence of feline mycobacterial disease and found that it is diagnosed at a rate of 1% of all routine feline submissions.

This is the context into which this thesis is presented; four Chapters (2, 3, 7 and 9) present significant new data which build on the currently accessible diagnostic capacity for companion animal mycobacterial infections in the UK, evaluating techniques that could be translated into clinical settings in both the near future but also points to more advanced methods which could form the next generation of diagnostic tests. These Chapters indicate that molecular diagnostics using formalin-fixed biopsy tissue is a sensitive method for the identification of TB; the test methodology developed and presented in Chapter 2 could readily be translated into a commercially available quantitative test and significantly improve the current diagnostic capacity for companion animal mycobacterial infections in the UK. Data presented in Chapter 7 also show the first attempt to develop *ante mortem* canine tests for TB; the development and implementation of the IGRA assay in real time during an outbreak evidenced its utility and it is

consequently already being offered to UK clinicians alongside the feline test at Biobest Laboratories, Edinburgh (Biobest, 2019).

Studies presented in this thesis are the first to develop and assess diagnostic serological assays for sub-clinical canine TB infections and in Chapter 8, the accuracy of a subset of the most promising tests is examined using Bayesian latent class analysis. The findings support the implementation of these tests and the possibility of using the comparative ELISA as a screening test for working dogs in order to allay fears that hunting hounds may be inadvertently contaminating farm land with *M. bovis* is currently under consideration by the Master of Foxhounds Association.

Two studies, presented in Chapters 3 and 9, explore the inflammatory processes associated with TB and other mycobacteria; one in cats and the other in dogs. Whilst the feline study was conducted on opportunistically collected blood samples, the canine study evaluated cytokine release into cell culture supernatant by cells stimulated with mycobacterial antigens. Despite the differences in study methodology, both studies demonstrate a strong cell mediated immune response to mycobacteria dominate in both of these species. Whilst these assays are currently too expensive to be considered for diagnostic use, the studies demonstrate considerable potential for the development of future multiplex cytokine assays which combine combinations of these inflammatory molecules into accurate and rapid diagnostic tests; in line with those being developed within human medicine, and for detection of TB in cattle (Clifford *et al.* 2019; Coad *et al.* 2019; Luo *et al.* 2019) but which are not currently available outside of a research context.

This thesis presents two unprecedented outbreaks of TB in companion animal species (Chapters 4 and 5); one canine and the other feline, both caused by the contamination of feedstuffs with *M. bovis* genotype 10:a. The canine outbreak, published in O'Halloran *et al.* 2018, was the first time that the transmission of *M. bovis* between dogs has been documented and resulted in nearly 100 dogs being euthanased. The origin of this outbreak was most likely to have been a contaminated bovine carcass which was fed inadvertently to the Foxhounds housed at the kennel. The findings of the outbreak investigation prompted a government policy

review of the feeding of raw products from fallen stock to hounds, permitted under Article 18 of Commission Regulation (EC) No. 1069/2009. A condition of this regulation is that fallen stock fed are not killed or have not died as a result of the presence or suspected presence of a disease communicable to humans or animals. An interpretation of this is that TB reactors, normally removed by APHA, and inconclusive reactors, should not be fed. The overall likelihood of at least one foxhound becoming infected with *M. bovis* within the next five years, if practices remained unchanged, was considered to be medium. In order to mitigate this risk, DEFRA has introduced tighter restrictions on the collection and feeding of fallen stock to hounds in registered kennels. As of 10 October 2017, legislation came into effect as a result of the findings presented in Chapter 4, to ban the feeding of offal from livestock species to dogs from recognised kennels or packs of hounds in England (Anon. 2017). Hunt kennel operators are also now required to carry out additional examinations for TB lesions in fallen stock originating from high risk premises. Additionally, APHA has developed training materials and guidance on the identification of TB in carcases of cattle and other livestock, for collectors and kennels feeding fallen stock to hounds.

The feline outbreak of *M. bovis* due to contaminated venison being fed raw to cats (Chapter 4) has highlighted a number of wider problems. It was clear from early in the investigations that were undertaken with the owners of cats affected by this outbreak that the public have little to no awareness of the microbiological risks presented to them or their pets by feeding raw food diets; a finding which is supported by the published literature. This is a deficit which urgently requires future work to address to redress the problem. Unlike the canine TB outbreak, these cats were affected in geographically disparate areas and countries of the UK and this meant that it was more challenging to unite different stakeholders around a unified approach to the outbreak, particularly as APHA do not have a statutory responsibility to investigate *M. bovis* infections in pets so initially no culture confirmation was offered *pro bono*. However, the conclusions of the epidemiological investigation were robust as they were evidenced by a number of culture isolates of *M. bovis* being whole genome sequenced, proving that they were genetically identical. As noted in the investigation, the withdrawal of UK Government funding to enable the culture high-risk companion animal samples in recent years

has led to owners showing a reluctance to choose this option. Negotiations are ongoing within the APHA as a result of the data presented within this thesis to potentially reinstate at least a portion of such funding to aid in the case of future outbreak situations. Owners who opted not to pay for culture testing were instead using tests viewed as un-validated by APHA and so future work is needed to increase the confidence in the accuracy of diagnostic tests for companion animal mycobacterial infections aside from culture.

As well as the devastating impact of both of these unparalleled outbreaks on the animals affected, significant attendant risks to public health also emerged which were investigated contemporaneously (Chapters 4 and 6). Jointly, these public health investigations identified five people with latent TB infections (LTBI); almost equal to the six cases of *M. bovis* transmitted from cats to humans in the last 150 years (Public Health England, 2014). Due to the latent state of these infections it is not possible to definitively prove that they were zoonotic in origin, but the risk is considered "plausible" and "real" (O'Halloran *et al.* 2018). The nature of these investigations are by their nature complex and publications included in this thesis sets out a robust formal methodology for the approach (Phipps *et al.* 2019). The protocol was developed during the canine outbreak and was then successfully utilised when the feline cases emerged. This highlights the importance of understanding the nature of the pathogen causing the disease to establish zoonotic risk and inform appropriate action.

Both of the outbreaks referred to above were caused by the same genotype of *M. bovis,* namely 10:a, and in both instances a number of cats and dogs were affected by severe and fulminant clinical disease which resulted in death or euthanasia on welfare grounds. This is clearly at odds with the conventional dogma in which TB is viewed as a chronic disease with an insidious onset before more severe clinical signs develop. Interestingly, in both instances pathological findings occurred which cannot be fully explained by our current knowledge of TB dynamics and kinetics in these species, such as the severe, necrotising renal lesions seen in a number of kennel dogs at *post mortem* examination. Chapter 11 of this thesis begins to characterise a novel *in vitro* method for the reliable generation of canine bone marrow derived macrophages using recombinant CSF-1. It is hoped that this work will now allow for the

characterisation of host-pathogen interactions between strains or subtypes *M. bovis* which have now been successfully isolated from animals affected by these outbreaks and canine bone marrow derived macrophages and the macrophage-like DH82 cell line.

Finally, Chapter 10 formally examines our current knowledge of canine TB and brings the published literature for this species in line with that which is currently the case for feline mycobacteriosis.

To conclude, this thesis has presented data which significantly add to our current knowledge with respect to the clinical aspects of TB in both cats and dogs in the UK. In the future, studies should focus on further understanding of the host-pathogen interaction. This will enable the development of additional diagnostic tests, or potentially vaccines that will enable the control of TB in companion animals

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Appendices

Appendix 1.1: IGRA results for the 164 hounds tested

Test number	Negative control $(\bar{x} + 2SD)$	Positive control $(\bar{x} - 2SD)$	PPDA $(\bar{x} - 2SD)$	PPDB $(\bar{x} - 2SD)$	ESAT-6/CFP-10 $(\bar{x} - 2SD)$	Significant Responses
1	0.05	3.41	-0.09	0.1	0	B only
2	0.09	2.11	0.03	0.05	0.29	EC only
3	0.12	0.65	0.17	0.13	0.13	Fail
4	0.01	3.99	0.06	0.36	0.36	B>A +E
5	0.03	4.11	0.48	1.62	0.96	B>A +E
6	0.09	2.59	0.23	0.69	0	B>A
7	0.01	3.73	0.05	0.3	0.77	B>A +E
8	0.2	3.05	-0.07	0.05	0.03	Negative
9	0.03	4.06	0.15	0.61	1.18	B>A +E
10	0.03	2.69	0.03	0.07	0	Negative
11	0.11	0.25	0.07	0.09	0.07	Fail
12	0.09	2.87	0.02	0	0	Negative
13	0.06	3.88	0.2	0.52	0.63	B>A +E
14	0.07	2.27	0.06	0.16	0	B only
15	0.08	2.27	0.05	0.06	0.08	Negative
16	0.04	0.2	0.01	0	0.05	Fail
17	0.3	3.51	0.45	0.3	0.5	A>B + E
18	0.18	2.8	0.13	0.13	0.23	B>A +E
19	0.01	4.25	0.17	0.44	2.24	B>A +E
20	0.03	4.05	0.06	0.03	0	Negative
21	0.09	2.11	0.03	0.05	0.29	EC only
22	0.13	1.79	0.1	0.1	0.1	Negative
23	0.32	2.8	0	0.1	0.09	Fail
24	0.07	3.16	0.08	0.14	0	B>A
25	0.08	3.34	0.06	0.21	0.03	B only
26	0.08	3.94	0.05	0.45	0.06	B only
27	0.1	2.84	0.5	1.5	0.8	B>A +E
28	0.16	3.35	0.03	0.04	0.07	Negative
29	0.04	3.65	1.4	1.68	1.89	B>A +E
30	0.14	3.5	0.36	0.71	2.56	B>A +E
31	0.05	2.76	0.08	0.14	0.24	B+E
32	0.15	2.18	0	0.12	0	Negative
33	0.3	3.02	0.12	0.04	0	Negative
34	0.25	3.03	0.01	0.04	0	Negative
35	0.12	3.29	0.05	0.81	0.71	B+E

36	0.08	1.99	0.01	0	0	Negative
37	0.02	3.05	0	0.25	0.21	B+E
38	0.07	3.33	0.06	0.07	0	Negative
39	0.18	2.8	0.13	0.13	0.23	B>A +E
40	0.07	2.48	0.01	0.03	0.08	EC only
41	0.25	1.34	0.03	0	0.18	Negative
42	0.12	4	0	0.01	0.01	Negative
43	0.04	0.71	0.04	0	0.03	Fail
44	0.08	0.26	0.05	0	0	Fail
45	0.05	4.62	0	0.51	0.21	B+E
46	0.09	2.18	0.06	0.08	0.14	EC only
65	0.06	4.51	0.07	0.04	-0.02	Negative
66	0.01	4.17	0.02	0.01	0.40	EC only
67	0.16	4.32	0.03	-0.18	0.08	Negative
68	0.26	4.47	0.00	0.04	-0.01	Negative
69	0.05	0.79	0.01	-0.20	0.46	EC only
70	0.06	2.00	0.03	-0.05	-0.03	Negative
71	0.08	2.22	0.06	0.02	0.02	Negative
72	0.02	4.60	0.01	-0.03	-0.01	Negative
73						Fail
74	0.13	0.65	0.01	0.04	0.00	Negative
75	-0.01	2.01	-0.01	0.00	-0.01	Negative
76	0.00	2.27	-0.01	-0.01	-0.01	Negative
77	0.01	4.51	0.00	0.08	0.00	Negative
78						Fail
79	0.01	2.44	-0.02	-0.03	-0.02	Negative
80	0.02	4.42	0.01	-0.01	-0.01	Negative
81	0.00	2.52	0.00	0.02	-0.01	Negative
82	0.82	3.91	0.18	0.29	0.07	Negative
83	0.00	3.34	0.00	0.00	-0.25	Negative
84	0.02	4.61	0.01	0.02	0.01	Negative
85	0.06	3.91	0.01	0.10	-0.01	B only
86	0.01	4.10	0.44	1.25	0.26	B>A +E
87	0.01	4.28	0.19	1.05	0.13	B>A +E
88	0.02	4.23	0.01	0.12	0.02	B only
89	0.03	4.24	0.03	0.18	0.00	B only
90	0.02	4.10	0.12	1.32	1.70	B>A +E
91						Fail
92	1.19	3.43	0.20	1.18	1.05	Fail
93	0.03	3.16	0.07	0.29	0.10	B>A +E
94	-0.01	0.86	-0.01	-0.01	-0.01	Negative
95	0.97	3.55	0.00	-0.01	0.00	Negative
96	0.02	3.66	0.03	0.04	0.02	Negative

97	0.01	4.73	0.10	0.11	0.00	B>A
98	0.01	4.30	0.01	0.95	0.01	B only
99	0.66	3.98	0.01	0.01	0.01	Negative
100	0.02	4.16	0.02	0.22	0.02	B only
101	0.02	4.15	-0.34	0.02	0.00	Negative
102	-0.06	4.42	-0.07	-0.08	-0.17	Negative
103	0.01	4.19	0.01	-0.13	0.02	Negative
104	0.08	2.73	-0.05	-0.04	-0.05	Negative
105	-0.06	4.44	-0.06	-0.08	-0.07	Negative
106	0.08	4.49	0.14	-0.03	-0.04	A only
107	0.00	4.42	-0.02	-0.05	-0.06	Negative
108	0.09	4.64	-0.01	-0.02	-0.04	Negative
109	0.11	4.51	0.03	-0.03	-0.03	Negative
110	0.04	4.39	0.01	0.01	-0.01	Negative
P1	0.15	1.11	0.09	0.09	0.05	Negative
P2	0.20	2.35	0.08	0.10	0.12	Negative
P3	0.39	3.48	0.16	0.13	0.13	Negative
111	0.26	0.62	0.08	0.31	0.17	Positive
112	0.49	2.05	0.09	0.09	0.07	Negative
113	0.14	0.86	0.10	0.09	0.08	Negative
114	0.34	1.79	0.08	0.11	0.06	Negative
115	0.12	1.85	0.14	0.17	0.11	Positive
116	0.19	2.98	0.13	0.13	0.10	Negative
117	0.14	3.37	0.09	0.12	0.11	Negative
118	0.14	2.97	0.08	0.20	0.15	Positive
119	0.11	3.13	0.11	1.18	0.48	Positive
120	0.18	2.59	0.16	0.71	0.49	Positive
121	0.16	1.17	0.07	0.13	0.06	Negative
122	0.26	4.07	0.11	0.27	0.31	Positive
123	0.17	4.01	0.11	0.19	0.25	Positive
124	0.20	4.00	0.14	0.05	0.14	Negative
125	0.17	3.43	0.14	0.25	0.14	Positive
126	0.26	4.13	0.13	0.16	0.14	Negative
127	0.21	4.42	0.39	1.44	0.41	Positive
128	0.16	3.91	0.20	0.94	1.59	Positive
129	0.16	3.80	0.14	0.28	0.18	Positive
130	0.14	3.68	0.40	0.42	0.21	Positive
131	0.19	4.14	0.22	1.37	0.57	Positive
132	0.20	3.62	0.20	0.10	0.05	Negative
133	0.13	3.19	0.31	1.51	0.33	Positive
134	0.13	2.84	0.09	0.09	0.10	Negative
135	0.13	1.95	0.25	0.16	0.10	Positive
136	0.15	2.56	0.00	0.16	0.11	Positive

137	0.24	3.15	0.19	0.16	0.15	Negative
138	0.13	2.54	0.11	0.18	0.13	Positive
139	0.32	2.87	0.12	0.09	0.09	Negative
140	0.32	3.38	0.18	0.17	0.37	Positive
141	0.15	2.41	0.13	0.14	0.09	Negative
142	0.15	2.30	-0.16	0.20	0.37	Positive
143	0.30	3.38	0.19	-0.05	-0.07	Negative
144	0.23	2.98	0.14	0.15	0.12	Negative
145	0.15	3.16	0.17	0.10	0.11	Positive
146	0.12	0.08	0.09	0.07	0.10	Fail
147	0.16	1.74	0.05	0.15	0.12	Negative
148	0.31	1.43	0.12	0.18	0.15	Negative
149	0.17	2.10	0.19	0.27	0.14	Positive
150	0.50	2.45	0.14	0.12	0.14	Negative
151	0.24	3.04	0.19	0.17	0.12	Negative
152	0.16	0.91	0.09	0.12	0.08	Negative
153	0.15	0.57	0.10	0.12	0.09	Negative
154	0.20	1.50	0.06	0.10	0.10	Negative
155	0.14	0.25	0.10	0.08	0.10	Negative
156	0.10	1.07	0.28	0.63	0.13	Positive
157	0.24	4.48	0.13	0.16	0.15	Negative
158	0.66	3.45	0.25	0.70	0.22	Positive
159	0.15	2.61	0.17	0.89	0.22	Positive
160	0.15	2.59	0.34	0.56	0.08	Positive
161	0.37	1.80	0.16	0.19	0.12	Negative
162	0.27	4.50	1.00	2.79	1.58	Positive
163	0.19	1.83	0.18	0.52	0.12	Positive
164	0.39	2.62	0.42	1.06	0.02	Positive
165	0.34	3.60	0.24	0.19	0.21	Negative
166	0.35	4.04	0.22	0.78	0.20	Positive
167	0.60	3.41	0.16	0.16	0.23	Negative
168	0.25	3.91	0.67	1.15	0.26	Positive
169	0.25	1.47	0.27	0.21	0.17	Positive
170	0.27	2.80	0.20	0.74	0.45	Positive
171	0.28	3.15	0.20	0.25	0.08	Negative
172	0.43	0.82	0.09	0.11	0.17	Negative
173	0.44	2.65	0.22	0.31	0.15	Negative
174	0.27	3.56	0.45	0.96	0.53	Positive
175	0.50	3.05	0.15	0.10	0.04	Negative
176	0.22	2.61	0.20	0.28	0.12	Positive
177	0.27	2.89	0.15	0.18	0.18	Negative
178	0.31	2.82	0.28	0.70	0.33	Positive
179	0.44	1.48	0.02	0.12	0.16	Negative

180	0.14	0.23	0.08	0.06	0.06	Negative
181	0.08	2.17	0.10	0.13	0.08	Positive
182	0.09	2.74	0.19	0.88	0.14	Positive
183	0.10	3.48	0.11	0.08	0.06	Positive
184	0.22	2.50	0.05	-0.08	0.04	Negative
185	0.27	2.65	0.08	0.12	0.11	Negative
186	0.17	3.49	0.81	0.73	0.25	Positive
187	0.11	2.35	0.10	0.10	0.08	Negative
188	0.09	2.60	-0.03	0.09	0.07	Negative
189	0.07	2.54	0.07	0.09	0.07	Positive
190	0.10	2.66	0.05	0.07	-0.01	Negative
Y1	0.19	3.20	0.12	0.09	0.09	Negative
Y2	0.13	3.95	0.07	0.09	0.10	Negative
Y3	0.37	2.13	0.12	0.14	0.13	Negative

Appendix 1.2: Serology results for the 164 hounds tested

Serology results of ChembioDPP VetTB Assay				
Test Number	MPB83 (Visible Assessment)	MPB83 (RLU)	ESAT-6/CFP-10 (Visual Assessment)	ESAT-6/CFP-10 (RLU)
1	Negative	8.01	Negative	0
2	Negative	1.31	Negative	0
3	Negative	2.04	Negative	0
4	Negative	0	Negative	0
5	Negative	3.51	Negative	0
6	Visible positive	127	Negative	0
7	Negative	0	Negative	0
8	Negative	2.43	Negative	0
9	Negative	0	Negative	0
10	Negative	1.22	Negative	0.9
11	Negative	0	Negative	0
12	Negative	0	Visible positive	88.75
13	Negative	0	Negative	0
14	Negative	0	Negative	0
15	Intermediate	73.62	Negative	0
16	Negative	0	Negative	0
17	Negative	0	Negative	0
18	Negative	0	Negative	0
19	Negative	0	Negative	0
20	Negative	0	Negative	0
21	Negative	0	Negative	0
22	Negative	0	Negative	0

23	Negative	1.54	Negative	0
24	Negative	15.56	Negative	0.65
25	Negative	0	Intermediate	18.54
26	Negative	0	Negative	0
27	Visible positive	389.21	Negative	0
28	Negative	0	Negative	0
29	Negative	0.89	Negative	0
30	Visible positive	297.47	Negative	3.46
31	Negative	5.79	Negative	0
32	Negative	8.59	Negative	0
33	Negative	0	Negative	0
34	Negative	6.79	Negative	0
35	Negative	0	Negative	0
36	Negative	9.42	Negative	0
37	Negative	0	Negative	0
38	Negative	0	Negative	0
39	Negative	0	Negative	0
40	Visible positive	146.04	Negative	0
41	Negative	0.7	Negative	0
42	Negative	1.23	Negative	0
43	Negative	1.59	Negative	0
44	Negative	11.31	Negative	0
45	Negative	0	Negative	0
46	Negativeative	12.71	Negativeative	0.93
47	Negativeative	0	Negativeative	0
48	Negativeative	0	Negativeative	0
49	Negativeative	17.72	Negativeative	0
50	Negativeative	0.6	Negativeative	0
51	Negativeative	0	Negativeative	3.69
52	Negativeative	0	Negativeative	0
53	Visible positive	124.43	Negativeative	0
54	Negativeative	0	Negativeative	0
55	Negativeative	16.33	Negativeative	0
56	Negativeative	0	Negativeative	0
57	Negativeative	17	Negativeative	0
58	Negativeative	0	Negativeative	0
59	Negativeative	0	Negativeative	0
60	Negativeative	11.73	Negativeative	0
61	Negativeative	0	Negativeative	0
62	Negativeative	21.97	Negativeative	0
63	Negativeative	0	Intermediate	34.66
64	Negativeative	6.61	Negativeative	0
65	Negativeative	9.07	Negativeative	0

66	Negativeative	5.04	Negativeative	0
67	Negativeative	0.98	Negativeative	0
68	Negativeative	5.33	Negativeative	0
69	Negativeative	10.51	Negativeative	0
70	Intermediate	58.72	Negativeative	2.32
71	Negativeative	12.14	Negativeative	0
72	Negativeative	7.78	Negativeative	0
73	Negativeative	6.86	Negativeative	0
74	Negativeative	0.71	Negativeative	0
75	Negativeative	0	Negativeative	3.9
76	Negativeative	2.05	Negativeative	0
77	Negativeative	1.16	Negativeative	0
78	Negativeative	9.58	Negativeative	0
79	Negativeative	1.93	Negativeative	0
80	Negativeative	0.9	Negativeative	0
81	Negativeative	0	Negativeative	0
82	Negativeative	0.23	Negativeative	0
83	Negativeative	10.54	Visible positive	113.65
84	Negativeative	13.82	Negativeative	0
85	Negativeative	20.39	Negativeative	0
86	Negativeative	6.13	Negativeative	0
87	Negativeative	21.99	Negativeative	0
88	Negativeative	0	Negativeative	0
89	Negativeative	0	Negativeative	0
90	Negativeative	3.22	Negativeative	0
91	Negativeative	14.56	Negativeative	0
92	Negativeative	0	Negativeative	0
93	Negativeative	0	Negativeative	0
94	Negativeative	0	Negativeative	0
95	Negativeative	4.63	Negativeative	4.07
96	Negativeative	2.15	Intermediate	10.64
97	Negativeative	6.28	Negativeative	0
98	Negativeative	0	Negativeative	0
99	Negativeative	2.68	Negativeative	0
100	Negativeative	0	Negativeative	0
101	Negativeative	20.84	Negativeative	0
102	Negativeative	0	Negativeative	0
103	Negativeative	17.41	Negativeative	0
104	Negativeative	0	Negativeative	0
105	Negativeative	2.16	Intermediate	8.94
106	Negativeative	0	Negativeative	0
107	Visible positive	4169.89	Negativeative	0
108	Negativeative	0	Negativeative	0

109	Negativeative	0	Negativeative	0
110	Negativeative	1.66	Negativeative	0
111	Negativeative	0	Intermediate	14.36
112	Negativeative	0	Negativeative	0
113	Negativeative	0	Negativeative	0
114	Negativeative	0	Negativeative	0
115	Negativeative	6.45	Negativeative	0
116	Negativeative	20.97	Negativeative	0
117	Negativeative	8.53	Negativeative	0
118	Negativeative	6.43	Negativeative	0
119	Visible positive	905.4	Negativeative	0
120	Negativeative	0	Negativeative	0
121	Negativeative	0	Negativeative	0
122	Negativeative	0	Negativeative	0
123	Negativeative	0	Intermediate	13.78
124	Negativeative	0	Negativeative	0
125	Negativeative	0	Negativeative	2.83
126	Negativeative	0	Intermediate	27.02
127	Negativeative	0	Negativeative	0
128	Negativeative	2.29	Negativeative	0
129	Negativeative	0	Negativeative	1.53
130	Negativeative	0	Negativeative	0
131	Negativeative	0	Negativeative	0
132	Negativeative	14.72	Negativeative	0
133	Negativeative	0.83	Negativeative	0
134	Negativeative	0	Negativeative	0
135	Negativeative	0	Negativeative	0
136	Negativeative	0	Negativeative	0
137	Negativeative	6.53	Negativeative	0
138	Negativeative	12.78	Negativeative	0
139	Negativeative	4.67	Negativeative	4.39
140	Negativeative	6.93	Negativeative	0
141	Negativeative	7.88	Negativeative	0
142	Negativeative	0	Negativeative	0
143	Negativeative	0	Negativeative	0
144	Negativeative	0	Negativeative	0
145	Negativeative	9.27	Negativeative	0
146	Negativeative	0	Negativeative	0
147	Negativeative	0	Negativeative	0
148	Negativeative	0	Negativeative	0
149	Negativeative	0	Negativeative	0
150	Negativeative	0	Negativeative	0
151	Negativeative	0	Negativeative	0

152	Negativeative	0	Negativeative	0
153	Negativeative	0	Negativeative	0
154	Negativeative	0	Negativeative	0
155	Negativeative	0	Negativeative	0
156	Negativeative	0	Negativeative	0
157	Visible positive	115.53	Negativeative	0
158	Negativeative	0	Negativeative	0
159	Visible positive	286.51	Negativeative	2.85
160	Negativeative	0	Negativeative	0
161	Negativeative	8.89	Negativeative	0
162	Negativeative	0	Negativeative	0
163	Negativeative	0.82	Negativeative	0
164	Negativeative	0	Negativeative	0
165	Negativeative	0	Negativeative	0
166	Negativeative	0	Negativeative	0
167	Negativeative	0	Negativeative	0
168	Negativeative	0	Negativeative	0
169	Negativeative	0	Negativeative	0
170	Negativeative	0	Negativeative	0

Appendix 2: WinBUGS syntax of Model One

```
Model{
X[1:16] ~ dmulti(p[1:16], n)
p[1] <- pi*(1-SeIGRA)*(1-SeCompPPD)*(1-SeDPP)*(1-SeIdexx) + (1-
pi)*SpIGRA*SpCompPPD*SpDPP*SpIdexx
p[2] <- pi*SeIGRA*(1-SeCompPPD)*(1-SeDPP)*(1-SeIdexx)+(1-pi)*(1-
SpIGRA)*SpCompPPD*SpDPP*SpIdexx
p[3] <- pi*SeIGRA*SeCompPPD*(1-SeDPP)*(1-SeIdexx)+(1-pi)*(1-SpIGRA)*(1-
SpCompPPD)*SpDPP*SpIdexx
p[4] <- pi*SeIGRA*SeCompPPD*SeDPP*SeIdexx + (1-pi)*(1-SpIGRA)*(1-SpCompPPD)*(1-
SpDPP)*(1-SpIdexx)
p[5] <- pi*(1-SeIGRA)*SeCompPPD*SeDPP*(1-SeIdexx) + (1-pi)*SpIGRA*(1-
SpCompPPD)*(1-SpDPP)*SpIdexx
p[6] <- pi*(1-SeIGRA)*(1-SeCompPPD)*(1-SeDPP)*SeIdexx + (1-
pi)*SpIGRA*SpCompPPD*SpDPP*(1-SpIdexx)
p[7] <- pi*(1-SeIGRA)*SeCompPPD*(1-SeDPP)*(1-SeIdexx) + (1-pi)*SpIGRA*(1-
SpCompPPD)*SpDPP*SpIdexx
p[8] <- pi*(1-SeIGRA)*(1-SeCompPPD)*SeDPP*(1-SeIdexx) + (1-
pi)*SpIGRA*SpCompPPD*(1-SpDPP)*SpIdexx
p[9] <- pi*SeIGRA*SeCompPPD*SeDPP*(1-SeIdexx) + (1-pi)*(1-SpIGRA)*(1-
SpCompPPD)*(1-SpDPP)*SpIdexx
p[10] <- pi*(1-SeIGRA)*(1-SeCompPPD)*SeDPP*SeIdexx + (1-
pi)*SpIGRA*SpCompPPD*(1-SpDPP)*(1-SpIdexx)
p[11] <- pi*SeIGRA*(1-SeCompPPD)*SeDPP*(1-SeIdexx)+ (1-pi)*(1-
SpIGRA)*SpCompPPD*(1-SpDPP)*SpIdexx
```

```
p[12] <- pi*SeIGRA*(1-SeCompPPD)*(1-SeDPP)*SeIdexx + (1-pi)*(1-
SpIGRA)*SpCompPPD*SpDPP*(1-SpIdexx)
p[13] <- pi*(1-SeIGRA)*SeCompPPD*SeDPP*SeIdexx + (1-pi)*SpIGRA*(1-
SpCompPPD)*(1-SpDPP)*(1-SpIdexx)
p[14] <- pi*SeIGRA*SeCompPPD*(1-SeDPP)*SeIdexx + (1-pi)*(1-SpIGRA)*(1-
SpCompPPD)*SpDPP*(1-SpIdexx)
p[15] <- pi*(1-SeIGRA)*SeCompPPD*(1-SeDPP)*SeIdexx + (1-pi)*SpIGRA*(1-
SpCompPPD)*SpDPP*(1-SpIdexx)
p[16] <- pi*SeIGRA*(1-SeCompPPD)*SeDPP*SeIdexx + (1-pi)*(1-
SpIGRA)*SpCompPPD*(1-SpDPP)*(1-SpIdexx)
pi ~ dbeta(1.0,1.0)
SeIGRA ~ dbeta (1.0,1.0)
SpIGRA ~ dbeta (1.0,1.0)
SeCompPPD ~ dbeta (1.0,1.0)
SpCompPPD ~ dbeta (1.0,1.0)
SeDPP ~ dbeta (1.0,1.0)
SpDPP ~ dbeta (1.0,1.0)
SeIdexx ~ dbeta (1.0,1.0)
SpIdexx ~ dbeta (1.0,1.0)
ppvIGRA <- pi*SeIGRA/(pi*SeIGRA+(1-pi)*(1-SpIGRA))
ppvCompPPD <- pi*SeCompPPD/(pi*SeCompPPD+(1-pi)*(1-SpCompPPD))
ppvDPP <- pi*SeDPP/(pi*SeDPP+(1-pi)*(1-SpDPP))
ppvIdexx <- pi*SeIdexx/(pi*SeIdexx+(1-pi)*(1-SpIdexx))
npvIGRA <- SpIGRA*(1-pi)/(SpIGRA*(1-pi)+pi*(1-SeIGRA))
npvCompPPD <- SpCompPPD*(1-pi)/(SpCompPPD*(1-pi)+pi*(1-SeCompPPD))
npvDPP <- SpDPP*(1-pi)/(SpDPP*(1-pi)+pi*(1-SeDPP))
npvIdexx <- SpIdexx*(1-pi)/(SpIdexx*(1-pi)+pi*(1-SeIdexx))
}
list(n=164, X=c(66,62,15,4,3,3,2,2,2,1,1,1,1,1,0,0))
```

Project Outcomes

Successful Grant Applications (Chronological order)

1. O'Halloran C. Investigating the Canine Macrophage Response to Mycobacteria.
   PetSavers Student Research Project Grant; £1000 March 2019 - September 2019.
2. O'Halloran C. The identification of antigen-specific biomarkers for the diagnosis of canine tuberculosis caused by *Mycobacterium bovis.*
   American Kennel Club Acorn Grant US$15,000; August 2018 - August 2019.
3. O'Halloran C. and Gunn-Moore, D.A. Validation of diagnostic assays for canine tuberculosis.
   Petplan Charitable Trust; £10,000 November 2017 – December 2018.

Publications (Chronological order)

1. **O'Halloran, C.,** Hope, J.C. and Gunn-Moore, D.A. (2019) Bayesian latent class estimation of performance parameters of diagnostic tests to identify *Mycobacterium bovis* infected domestic dogs (*Canis lupus familiaris*). *In preparation.*
2. Brindley, F., Whitbread, T., and **O'Halloran, C.** (2019) First confirmed case of canine leproid granuloma (CLG) in a UK dog. *In preparation.*
3. **O'Halloran, C.,** Černá, P., Breheny, C., Reed, N., Simpson, K., Cade, S., Jones, J., Brown, R., Slade, S., Papasouliotis, K. and Gunn-Moore, D.A. (2019) Investigation of pathological haemorrhage in Maine Coon cats. Veterinary Record. *Under Review.*
4. **O'Halloran, C.,** Burr, P., McDonald, K., Rhodes, S., Gunn-Moore, D.A. and Hope, J.C. (2019) Comparative performance of ante-mortem diagnostic assays for the identification of Mycobacterium bovis-infected domestic dogs (*Canis lupus familiaris*). Scientific Reports *Under Review.*

5. Stavinohova, R., **O'Halloran, C.,** Newton, R., Oliver, J., Scurrell, E., and Gunn-Moore, D.A. (2019) Histopathological and clinical features of feline ocular mycobacteriosis in the UK: a multicentre retrospective case series. Veterinary Pathology. *In Press.*
6. **O'Halloran, C.,** Ioannidi, O., Reed, N., Murtagh, K., Hope, J., Gale, J., Burr, P., Dettemering, E., Van Poucke, S., Vickers, J., Dobromylskyj, M., Mitchell, J., Gascoyne-Binzi, D., Howe, R. and Gunn-Moore, D.A. (2019) Tuberculosis due to *Mycobacterium bovis* in pet cats associated with feeding a commercial raw food diet. Journal of Feline Medicine and Surgery *In Press.*
7. Sharp, E., Taylor, S. and **O'Halloran, C.** (2019) Unusual presentation of canine *Mycobacterium avium* infection Veterinary Record. 184, 800. DOI: 10.1136/vr.105311
8. **O'Halloran, C.,** Gunn-Moore D.A., Reed, N., Vickers, J., Dettmering, E., Ioannidi, O., and Murtagh, K. (2018) *Mycobacterium bovis* in pet cats. Veterinary Record. 183(16):510. DOI: 10.1136/vr.k4452.
9. **O'Halloran, C.,** McCulloch, L., Rentoul, L., Alexander, J., Hope, J.C. and Gunn-Moore, D.A. (2018) Cytokine and Chemokine Concentrations as Biomarkers of Feline Mycobacteriosis. Scientific Reports, 23;8 (1):17314. DOI: 10.1038/s41598-018-35571-5.
10. **O'Halloran, C.,** Hope, J.C. and Gunn-Moore D.A (2018) *Mycobacterium bovis* infection in working foxhounds. Veterinary Record, 22, 183(11):356 DOI:10.1136/vr.k3955.
11. Phipps, E., McPhedran, K., Edwards, D., Russell, K., O'Connor, C.M., Morris, J., **O'Halloran, C.** and Gunn-Moore D.A. (2018) *Mycobacterium bovis* tuberculosis in hunting hounds. Veterinary Record, 22, 183(11):356 DOI: 10.1136/vr.k3962
12. Černá, P., **O'Halloran, C.,** SjatkovskaJ, O. and Gunn-Moore, D.A. (2018) Outbreak of tuberculosis caused by *Mycobacterium bovis* in a cattery of Abyssinian cats in Italy Transboundary and Emerging Diseases. *In Press.*

13. Phipps, E., McPhedran, K., Edwards, D., Dampney, R., Russell, K., O'Connor, C., Gunn-Moore, D.A, **O'Halloran, C.,** Roberts, T. and Morris, J. (2018) Bovine tuberculosis in working foxhounds: lessons learned from a complex public health investigation Epidemiology and Infection, 9, 1-6 DOI: 10.1017/S0950268818002753
14. **O'Halloran, C.,** Hope, J.C., Dobromylskyj, M., Burr, P., McDonald, K., Rhodes, S., Roberts, T., Dampney R, De la Rua-Domenech, R., Robinson, N. and Gunn-Moore, D.A (2018) An outbreak of tuberculosis due to Mycobacterium bovis infection in a pack of English Foxhounds (2016-2017). Transboundary and Emerging Infectious Diseases, 65(6), 1872-1884 DOI:10.1111/tbed.12969
15. **O'Halloran, C.** and Gunn-Moore, D.A (2017) Mycobacteria in cats: An update In Practice, 39 (9), 399-406 DOI: 10.1136/inp.j4155
16. Breheny, C.R., Fox, V., Tamborini, A., **O'Halloran, C.,** Robertson, E., Cazzini, P., Birn-Jeffery, D., Henkin, J., Schwartz, T., Scase, T., Powell, R. and Gunn-Moore D.A. (2017) Novel characteristics identified in two cases of feline cowpox virus infection. JFMS Open Reports 11;3(2):2055116917717191. DOI: 10.1177/2055116917717191
17. **O'Halloran, C.** and Dobromylskyj, M. (2017) Clinical Mycobacterial Diseases of Companion Animals: Part 2 Companion Animal 22 (11) 2-7.
18. **O'Halloran, C.,** Major, A., Holmes, A., Lalor, S., Littler, R., Spence, S., Schwarz, T. and Gunn-Moore, D.A. (2017) Use of computed tomography imaging during long-term follow-up of nine feline tuberculosis cases. Journal of Feline Medicine and Surgery. DOI:1098612X1769947
19. Gunn-Moore, D.A. and **O'Halloran, C.** (2017) Hunting dogs and bovine TB. Veterinary Record. 180, 25: 616
20. Lalor, S. M., Clarke, S., Pink, J., Parry, A., Scurrell, E., Fitzpatrick, N., Watson, F., **O'Halloran, C.** and Gunn-Moore, D. (2017) Tuberculosis joint infections in four domestic cats Journal of Feline Medicine and Surgery Open Reports. 3, 2. DOI:2055116917719401

21. **O'Halloran, C.** and Gunn-Moore, D.A. (2016) TB in companion animals: assessing the zoonotic risk Official Vet Magazine 4: 16-19.
22. **O'Halloran, C.** and Dobromylskyj, M. (2016) Clinical Mycobacterial Diseases of Companion Animals: Part 1 Companion Animal 22 (6) 325-329.
23. **O'Halloran, C.,** Gunn-Moore, D.A. and Hope, J.C. (2016) Diagnosis of Feline Mycobacteriosis Veterinary Record 178 (6) 145. DOI: 10.1136/vr.i686.
24. **O'Halloran, C.,** Del-Pozo, J., Breheny, C., Gunn-Moore, D.A., Dobromylskyj, M., Papasoulitis, K., Simpson, K. and Henken, J. (2016) Unusual presentations of feline cowpox. Vet Rec. 29;179(17):442-443. DOI: 10.1136/vr.i5767.

3. Conference Presentations (Oral)

1. Official Veterinarian Conference, Swindon, UK, September 2019. "Raw diets: *Mycobacterium bovis* outbreaks in companion animals"
2. Institute of Biomedical Science Congress, Birmingham, UK, September 2019. "Histopathological insights into feline TB".
3. International Veterinary Immunology Symposium (IVIS), Seattle, USA, August 2019. "Characterisation of the companion animal immune response to *Mycobacterium bovis* infection using cytokine profiling."
4. International Society for Companion Animal Infectious Diseases (ISCAID), Portland, USA, October 2018. "Development of a diagnostic PCR assay for feline mycobacterial disease."
5. International Society for Companion Animal Infectious Diseases (ISCAID), Portland, USA, October 2018. "Circulating cytokine and chemokine concentrations as biomarkers of feline mycobacteriosis".
6. Feline Mycobacteriosis CPD; Finn Pathlogists, June 2016. "The immunology of companion animal mycobacterial diseases".

4. Conference Presentations (Abstract Posters)

1. **O'Halloran, C.,** Woods, G., Hope, J.C. and Gunn-Moore, D.A. (2019) Outbreak of *Mycobacterium bovis* tuberculosis in UK indoor-only cats associated with raw food feeding. International Society of Feline Medicine (ISFM) European Congress, Cavtat, Croatia.

   Winner of the Delegates' Choice Award for best poster.
2. Woods, G., **O'Halloran, C**. and Gunn-Moore, D.A. (2019) *Mycobacterium bovis* tuberculosis in an indoor cat within Scotland associated with commercial raw food. International Society of Feline Medicine (ISFM) European Congress, Cavtat, Croatia
3. **O'Halloran, C.,** Černá, P., Breheny, C., Reed, N., Simpson, K., Cade, S., Jones, J., Brown, R., Slade, S., Papasouliotis, K. and Gunn-Moore, D.A. (2019) Investigation of pathological haemorrhage in Maine Coon cats.

   International Society of Feline Medicine (ISFM) European Congress, Cavtat, Croatia.
4. **O'Halloran, C.,** Hope, J.C.. and Gunn-Moore, D.A (2018) Evaluation of immunodiagnostic assays for the diagnosis of *Mycobacterium bovis* infection in dogs. International Society for Companion Animal Infectious Diseases (ISCAID), Portland, USA
5. **O'Halloran, C.,** Hope, J.C., Dobromylskyj, M., Burr, P., McDonald, K., Rhodes, S., Roberts, T., Dampney, R., De la Rua-Domenech, R., Robinson, N. and Gunn-Moore, D.A. (2018) Tuberculosis due to *Mycobacterium bovis* infection in a pack of English foxhounds – where did it come from?

   International Society for Companion Animal Infectious Diseases (ISCAID), Portland, USA
6. Phipps, E., McPhedran, K., Edwards, D., Dampney, R., Russell, K., O'Connor, C., Gunn-Moore, D.A., **O'Halloran, C.,** Roberts, T. and Morris J. (2018) Bovine tuberculosis in working foxhounds: lessons learned from a complex public health investigation.

International Society for Companion Animal Infectious Diseases (ISCAID), Portland, USA

7. **O'Halloran, C.,** Hope, J.C., Dobromylskyj, M., Burr, P., McDonald, K., Rhodes, S., Roberts, T., Dampney R, De la Rua-Domenech, R., Robinson, N. and Gunn-Moore, D.A (2018) An outbreak of tuberculosis due to *Mycobacterium bovis* infection in a pack of English foxhounds (2016-2017).
   International Society for Companion Animal Infectious Diseases (ISCAID), Portland, USA
8. **O'Halloran, C.,** Hope, J.C.. Dobromylskyj, M. and Gunn-Moore, D.A (2018) Development of a diagnostic PCR assay for feline mycobacterial disease
   International Society for Companion Animal Infectious Diseases (ISCAID), Portland, USA
9. **O'Halloran, C.,** McCulloch, L., Rentoul, L., Hope, J.C., and Gunn-Moore, D.A. (2018) Circulating cytokine and chemokine concentrations as biomarkers of feline mycobacteriosis.
   International Society for Companion Animal Infectious Diseases (ISCAID), Portland, USA
10. Černá, P., **O'Halloran, C.,** SjatkovskaJ, O., Gunn-Moore, D.A. (2018) Outbreak of tuberculosis caused by *Mycobacterium bovis* in a cattery of Abyssinian cats in Italy.
    International Society of Feline Medicine (ISFM) European Congress, Sorrento, Italy.
11. **O'Halloran, C.,** Hope, J.C.. Dobromylskyj, M. and Gunn-Moore, D.A (2018) Development of a rapid PCR diagnostic test for feline mycobacterial disease using formalin-fixed paraffin-embedded tissue biopsies.
    International Society of Feline Medicine (ISFM) European Congress, Sorrento, Italy.
12. Mitchell, J.L., Street, S.N., **O'Halloran, C.,** Woolley, C.S., Hope, J.C. and Gunn-Moore, D.A. (2017) Differences in numbers of observable mycobacteria by Ziehl-Neelsen staining in skin and lymph node biopsy samples in cases of feline tuberculosis.
    International Society of Feline Medicine (ISFM) European Congress, Sorrento, Italy.

13. **O'Halloran, C.,** McCulloch, L., Rentoul, L., Alexander, J., Hope, J.C. and Gunn-Moore, D.A. (2016) Comparative Analysis of ciculating cytokine and chemokine concentreations as biomarkers of feline mycobacteriosis.

    British Society of Immunology (BSI) Congress, Liverpool, UK.

    Shortlisted for best poster presentation prize.

14. **O'Halloran, C.,** Hope, J.C and Gunn-Moore, D.A. (2016) Canine Tuberculosis: Forgotten but not gone?

    International Society for Companion Animal Infectious Diseases (ISCAID) Symposium, Bristol, UK.