
THE UNIVERSITY
*of* EDINBURGH

# Development of multi-parametric tests for the diagnosis of feline tuberculosis

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# Declaration

I declare that the work presented in this thesis has been completed by myself, or I have made a significant contribution to the work. In Chapter 2, Ziehl-Neelsen staining for all cases was performed by Laura MacDougall (Royal Veterinary College) under my supervision. In Chapter 3, haematoxylin and eosin staining and Ziehl-Neelsen staining was performed by Charlotte S C Woolley (The Roslin Institute) for eight cases, and Richa Dheendsa (Royal (Dick) School of Veterinary Studies) performed haematoylin and eosin, Ziehl-Neelsen, and immunohistochemical staining for four cases under my supervision. For all cases in Chapters 2 and 3, Masson's Trichrome staining was performed by the Veterinary Pathology Unit (Royal (Dick) School of Veterinary Studies). This work has not been previously submitted, in whole or in part, for any other degree of professional qualification. All the work and publications presented in this thesis are my own work, except where indicated. Where publications are included, I confirm that as the author I retain the copyright for such articles, or I have been granted permission by the copyright holder to reproduce the text provided the published article is appropriately referenced.

Jordan L. Mitchell

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# Project Outputs

## Publications

1. **Mitchell, J.**, O'Halloran, C., Hope, J. & Gunn-Moore, D. 2021. Late-presenting cases of commercial raw food-associated TB in cats in the UK. *Veterinary Record*. 189(3):118-119. doi: 10.1002/vetr.805

2. **Mitchell, J. L.***, Raper, A., Gunn-Moore, D. A. & Hope, J. C. 2021. Recognition of recombinant interferon-gamma from Felidae species by anti-cat antibodies. *Veterinary Immunology and Immunopathology*. 241:110327. doi: 10.1016/j.vetimm.2021.110327

3. **Mitchell, J. L.***, Stanley, P., McDonald, K., Burr, P., Rhodes, S. G., Gunn-Moore, D. A. & Hope, J. C. 2021. Diagnostic accuracy of the interferon-gamma release assay (IGRA) for cases of feline mycobacteriosis. *Preventive Veterinary Medicine*. 193:105409. doi: 10.1016/j.prevetmed.2021.105409

4. **Mitchell, J. L.***, Del Pozo, J., Woolley, C. S. C., Dheendsa, R., Hope, J. C. & Gunn-Moore, D. A. 2021. Histological and immunohistochemical features suggesting aetiological differences in lymph node and (muco)cutaneous feline tuberculosis lesions. *Journal of Small Animal Practice*. Online ahead of print. doi: 10.1111/jsap13386

5. **Mitchell, J. L.*,** O'Halloran, C., Stanley, P., McDonald, K., Burr, P., Gunn-Moore, D. A. & Hope, J. C. 2021. Serial interferon-gamma release assay (IGRA) testing to monitor treatment responses in cases of feline mycobacterisis. *Pathogens*. 10(6):657. doi: 10.3990/pathogens10060657

6. Černa, P.*, **Mitchell, J. L.§**, Lodzinska, J., Cazzini, P., Varjonen, K. & Gunn-Moore, D. A. 2020. Systemic *Mycobacterium kansasii* infection in two related cats. *Pathogens*. 9(11):959. doi: 10.3390/pathogens9110959

7. O'Halloran, C., Tørnqvist-Johnsen, C., Woods, G., **Mitchell, J.**, Reed, N., Burr, P., Gascoyne-Binzi, D., Wegg, M., Beardall, S., Hope, J. & Gunn-Moore, D. 2020. Feline tuberculosis caused by *Mycobacterium bovis* infection of domestic UK cats associated with feeding a commercial raw food diet. *Transboundary and Emerging Diseases*. Online ahead of print. doi: 10.1111/tbed.13889

8. **Mitchell, J. L.***, Ganis, L., Blacklock, B. T., Petrushkin, H., Hope, J. C. & Gunn-Moore, D. A. 2020. Ocular tuberculosis: More than 'Of Mice and Men'. *Ocular Immunology and Inflammation*. Online ahead of print. doi: 10.1080/09273948.2020.1797116

9. **Mitchell, J.**, Gunn-Moore, D. & Burr, P. 2019. Feline mycobacterial infections. *Veterinary Record*. 185(11):347-348. doi: 10.1136/vr.l5594

10. O'Halloran, C., Ioannidi, O., Reed, N., Murtagh, K., Dettemering, E., Van Poucke, S., Gale, J., Burr, P., Gascoyne-Binzi, D., Howe, R., Dobromylskyj, M. J., **Mitchell, J.**, Hope, J. & Gunn-Moore, D. 2019. Tuberculosis due to *Mycobacterium bovis* in pet cats associated with feeding a commercial raw food diet. *Journal of Feline Medicine and Surgery*. 21(8):667-681. doi: 10.1177/1098612X19848455

11. **Mitchell, J. L.** & Gunn-Moore, D. A. 2019. Mycobacterial infections of cats and dogs. *Veterinary Nursing Journal*. 34(4):102-107. doi: 10.1080/17415349.2018.1551103

* = presented in this thesis

§ = joint credit as first author

## Manuscripts Under Review and In Preparation

1. **Mitchell, J. L.¶**, MacDougall, L., Dobromylskyj, M. J., Smith, K., Stavinohova, R., Gunn-Moore, D. A., Hope, J. C. & Scurrell, E. Ocular mycobacterial lesions in cats. *Veterinary Pathology*. Under review

2. Tan, Y., Marques, A., **Mitchell, J. L.**, Liuti, T. & Schwarz, T. Computed Tomographic Sialography findings in 22 dogs surgically confirmed sialoceles. *Veterinary Radiology and Ultrasound*. Under review

3. Israeliantz, N., Massidda, P. A., **Mitchell, J. L.**, Richardson, J., Blacklock, B. & Liuti, T. Ocular biometry by computed tomography in rabbits. *Veterinary Ophthalmology*. In preparation

¶ = reformatted and presented in this thesis

## Oral Presentations

1. **Mitchell, J. L.** 2021. *Mycobacterial lesions in cats*. British Society of Veterinary Pathology, 27 October, online.

2. **Mitchell, J. L.** 2021. *Mycobacteriosis in Felidae: Generation of recombinant interferon-gamma from different Felidae species, and recognition by cat-specific anti-interferon-gamma antibodies*. 7th European Veterinary Immunology Workshop, 29-31 August, online.

3. **Mitchell, J. L.** 2020. *Diagnostic Accuracy of the Interferon-Gamma Release Assay for Diagnosing Cases of Feline Mycobacteriosis*. International Society for Companion Animal Infectious Diseases, 10-11 September, online.

Received the Idexx/ISCAID Award (Second Prize) for this presentation

4. **Mitchell, J. L.** 2019. *From Summerhall to Sorrento: The Tale of an Early Career Veterinary Researcher*. 3rd National Student Veterinary Research Conference. 2 November, Edinburgh, UK.

## Poster Presentations

1. **Mitchell, J. L.**, O'Halloran, C., Stanley, P., McDonald, K., Burr, P., Hope, J. C. & Gunn-Moore, D. A. 2021. *Serial interferon-gamma release assay (IGRA) testing to monitor treatment responses in cases of feline mycobacteriosis*. International Society of Feline Medicine Congress, 25-27 June. Online.

2. **Mitchell, J. L.**, Hope, J. C. & Gunn-Moore, D. A. 2019. *Comparison of histopathological features in feline tuberculosis lesions*. International Society of Feline Medicine Congress, 26-30 June. Cavtat, Croatia.

3. **Mitchell, J. L.**, Street, S. N., O'Halloran, C., Woolley, C. S. C., Hope, J. C. & Gunn-Moore, D. A. 2018. *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 Congress, 28 June-1 July. Sorrento, Italy.

# Abstract

Mycobacterial infections cause a substantial burden of disease in both human and animal populations and continue to provide a challenge in terms of both their diagnosis and treatment. Feline mycobacterial infections are increasingly recognised by veterinary staff but obtaining a rapid and reliable diagnosis in these cases remains problematic. Tuberculosis (TB) is the most frequently diagnosed mycobacterial disease in cats in Great Britain, where it is caused by infection with either *Mycobacterium* (*M.*) *bovis* or *M. microti*. Non-tuberculous mycobacteria also cause disease in cats, but a species-level diagnosis of mycobacterial infection cannot be accomplished on clinical signs alone. Accurately diagnosing these infections is of importance so that appropriate treatment can be initiated, and to ascertain the risk posed to humans due to the zoonotic potential of some species of mycobacteria, namely *M. bovis*. Specialist mycobacterial culture is limited by the fact it can take several months to attain a positive result, and that some species of mycobacteria are near-impossible to grow in the laboratory. Commercially available polymerase chain reaction (PCR) assays can be financially restrictive, especially for obtaining a species-level diagnosis in cases of TB, and extracting sufficient high-quality deoxyribonucleic acid (DNA) from formalin-fixed paraffin-embedded (FFPE) tissue samples for PCR testing can be a limiting factor. Finally, while the feline-specific interferon-gamma (IFNγ) release assay (IGRA) has been in use for over six years, there is a lack of data assessing the performance of this test. Therefore, there is plentiful scope for the advancement of diagnostics for cases of feline mycobacteriosis, especially feline TB, which would be of benefit for both cats and humans.

Ocular TB (OTB) is an unusual presentation of disease in humans and remains controversial in terms of its pathogenesis and treatment; OTB has also been reported in cats. Given this, it was hypothesised that the cat may provide insights into human OTB, but further work was needed to characterise feline ocular mycobacterial lesions. This study characterised the histopathological changes present in 24 cases of feline ocular mycobacteriosis, and described the immune cells present using immunohistochemistry. Granulomatous to pyogranulomatous choroidal lesions with concurrent retinitis and retinal detachment was the most common finding, but many choroidal lesions also showed infiltration with a substantial number of B-lymphocytes. Mycobacteria were identified in 83% of globes, sometimes in large numbers, in contrast with data from humans.

Histopathology is often the first diagnostic step performed that raises the suspicion of mycobacterial infection in cats. However, descriptions of the histopathological features of these lesions and characterisation of the cell populations present are lacking, particularly for cases of TB. One aim of this study was to identify whether differences in the appearance and composition of feline (muco)cutaneous and lymph node TB lesions could discern between infection with *M. bovis* or *M. microti*. A novel, descriptive scoring system was devised which suggested that the type of granuloma present differed between cases of *M. bovis* and *M. microti*, and this may provide a means of differentiating between these two aetiological agents at an earlier stage of the diagnostic investigation. It was also shown that

cases of TB can present with abundant acid-fast bacilli (AFB) on Ziehl-Neelsen staining; therefore, identification of large numbers of AFB does not rule out infection with tuberculous pathogens.

The next aim of this study was to assess the performance of tests of cell-mediated immunity, namely the IGRA, for the diagnosis of feline mycobacteriosis as well as the utility of the IGRA for monitoring treatment responses. Receiver-operating characteristic curve analysis suggested reducing test cut-off values that would improve sensitivity of the IGRA, in particular for cases of *M. bovis* infection, while maintaining test specificity. Adopting these adjusted thresholds also improved test agreement between the IGRA and culture and/or PCR-confirmed cases of mycobacteriosis. Assessment of IGRA responses in cats treated for mycobacterial infection showed that 78% of cats remain positive at the point of clinical resolution. Additionally, remaining positive at the end-of-treatment IGRA was not associated with recurrence of disease. Therefore, the IGRA is both a sensitive and specific test for the diagnosis of feline mycobacterial infections, especially for cases of TB, but based on these data it should not be recommended as a tool for monitoring responses to treatment.

The IGRA is an example of a test that has been successfully adapted for use in multiple species; the feline-specific IGRA has also been used in other felids, namely the lion. However, data to confirm that anti-cat IFNγ antibodies can bind to IFNγ from other species is lacking. A combined bioinformatics and bench-work approach was undertaken to identify, compare and characterise IFNγ from Felidae species, and then produce recombinant IFNγ (rIFNγ) from these species to determine cross-reactivity of anti-cat IFNγ antibodies. Cross-reactivity of Felidae rIFNγ with anti-dog and anti-bovine IFNγ antibodies was also performed. Anti-cat IFNγ antibodies successfully cross-reacted with five rIFNγ Felidae proteins. By extension, the feline-specific IGRA may be applicable for use in other Felidae, overcoming the limitation of a lack of specific reagents for use of diagnostic tests for TB in wildlife species.

Tests of humoral immunity for the diagnosis of TB are controversial in human medicine. While serodiagnostic tests have been developed for diagnosing TB in a range of animal species, the use of these tests in cats has been somewhat overlooked. To that end, an enzyme-linked immunosorbent assay for the comparative response between purified protein derivative (PPD) from *M. bovis* and PPD from *M. avium* for the diagnosis of feline TB was developed as part of this study. This assay had a sensitivity of 41% and specificity of 100%. Notably, when applied to a larger cohort of samples, a positive comparative PPD response was documented in a small number of cats that had histopathological changes suggestive of mycobacterial infection but were negative on culture, PCR and/or IGRA. This would suggest there is some scope of antibody-based diagnostics for identifying cases of feline TB.

Molecular-based diagnostics such as PCR allow for the direct identification of mycobacteria, rather than assessing for an indirect response of host immunity. Given the difficulties in extracting DNA from FFPE tissue samples for conventional PCR testing, a quantitative PCR (qPCR) for use on FFPE tissues was developed. Mycobacterial DNA was successfully amplified in 79% of samples. The TB-complex specific gene IS*6110* was detected in 64% of FFPE samples from cases of culture- or PCR-diagnosed TB. In

cases of *M. microti* infection, DNA for the region of difference (RD)12 portion of the genome was identified in six out of nine cats. Detection of RD1 DNA was only achieved in three out of ten cases of infection with *M. bovis*. For the identification of NTM species of importance in cats, novel primer/probe sets were designed against the genes IS*1311* and MLM_3300; these detect the presence of *M. avium* and *M. lepraemurium*, respectively. Four out of six samples from cats with *M. avium* infection were positive for IS*1311*; the two IS*1311* negative samples were positive for MLM_3300, suggestive of *M. lepraemurium* infection. Three of the four *M. lepraemurium* samples were positive for the primer/probe set targeting this species-specific gene. These data show that further work is required for the development of a qPCR assay to diagnose cases of TB from FFPE tissues, but in its current format it may be more successful for the identification of infection with some NTM species.

Overall, the work presented in this thesis advances our knowledge of the underlying immunopathology of feline mycobacterial infections, particularly for cases of TB. These results allow for the improvement of current diagnostic tests available for cases of feline mycobacteriosis, and the potential application of the IGRA for use in other felid species. The data also suggests a potential role for the use of antibody-based tests for diagnosing TB and provides further advancement of molecular approaches using qPCR.

# Lay Summary

Mycobacterial infections cause disease in both human and animal populations alike, yet they can be difficult to diagnose and treat. Cats can become infected with different species of mycobacteria; infection with *Mycobacterium* (*M.*) *bovis* or *M. microti* causes tuberculosis (TB), which is the most diagnosed mycobacterial disease in cats in Great Britain. Other mycobacterial infections in cats are due to species of bacteria termed non-tuberculous mycobacteria. It is important to identify which species of mycobacteria is causing disease in any particular cat because of the different treatment protocols required, and the fact that in a very small number of cases cats have been shown to infect humans. All reported cases of cat-to-human transmission of TB were due to infection with *M. bovis*. It is not possible to determine which species of mycobacteria is responsible for disease based on the clinical signs alone; therefore, further diagnostic testing is required. However, there are limitations to the tests currently employed. This work presented in this thesis aims to improve our ability to identify and diagnose mycobacterial infections in cats, and to determine whether some of these tests could be used in other felid species, such as lions.

This thesis describes the appearance and composition of mycobacterial lesions in the eye, skin and lymph nodes using a variety of different stains. The results of this study suggest differences exist in the appearance of TB lesions between *M. bovis* and *M. microti*. This thesis provides a critical evaluation of a blood-based test used to diagnose mycobacterial infections in cats, suggesting changes to test cut-off values which could improve the performance of this assay. It also suggests that this test is of limited benefit for follow-up testing to determine whether a cat has been successfully treated. The cat-specific antibodies used in this test that detect a protein produced by immune cells in cases of feline mycobacterial infection can bind to the equivalent protein produced in other felid species. Two novel diagnostic tests are presented; one that looks for the presence of specific antibodies in blood for the diagnosis of TB in cats, and a second test that looks for mycobacterial deoxyribonucleic acid (DNA) in tissue biopsy samples and how this can be used to identify specific species of mycobacteria.

Overall, the work presented in this thesis advances our knowledge of mycobacterial infections in cats. These results appear to be particularly useful for improving our ability to diagnose cases of TB in cats, as well as suggesting new ways by which this could be achieved.

# List of Abbreviations

| | |
|---|---|
| 1,25-$(OH)_2D$ | 1,25-hydroxyvitamin D |
| 25(OH)D | 25-hydroxyvitamin D |
| 3D | Three dimensional |
| 6FAM | 6-carboxyfluoroscein |
| ΔPPD | Comparative purified protein derivative |
| ΔRn | Magnitude of fluorescence signal |
| ACDP | Advisory Committee on Dangerous Pathogens |
| AFB | Acid-fast bacilli |
| AHVLA | Animal Health and Veterinary Laboratories Agency |
| ALS | Antibody-in-lymphocyte supernatant |
| APCs | Antigen-presenting cells |
| APHA | Animal and Plant Health Agency |
| AUC | Area under the curve |
| BAL | Bronchoalveolar lavage |
| BCA | B-cell attracting chemokine |
| BCG | Bacillus Calmette Guérin |
| BI | Bacillary index |
| bp | Base pairs |
| BSH | British Shorthair |
| bTB | Bovine tuberculosis |
| *C.* | *Candidatus* |
| C-BC | Carbonate-bicarbonate |
| CCL | C-C motif ligand |
| CD | Cluster of differentiation |
| cds | Coding deoxyribonucleic acid sequence |
| CFP-10 | Culture filtrate protein 10kDa |
| CI | Confidence interval |
| CINC | Cytokine-induced neutrophil chemoattractant |
| CMI | Cell-mediated immunity |
| CNS | Central nervous system |
| CT | Computed tomography |
| $C_t$ | Cycle threshold |
| CV | Coefficient of variation |
| CXCL | CXC motif chemokine ligand |
| CYP3A4 | Cytochrome P450 3A4 |
| DAB | 3,3'-diaminobenzidine |

| | |
|---|---|
| DLH | Domestic longhair |
| DNA | Deoxyribonucleic acid |
| DPBS | Dulbecco's phosphate buffered saline |
| DR | Direct repeat |
| DSH | Domestic shorthair |
| DTH | Delayed-type hypersensitivity |
| DxH | Domestic short/medium/longhair |
| E6C10 | Early secreted antigenic target 6kDa/culture filtrate protein 10kDa |
| EDTA | Eythlene diamine tetra-acetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| ELISPOT | Enzyme-linked immunosorbent spot |
| ESAT-6 | Early secreted antigenic target 6kDa |
| F- | Female, unknown neuter status |
| Fc | Crystallisable fragment |
| FeLV | Feline leukaemia virus |
| FF | Fite-Faraco |
| FFPE | Formalin-fixed paraffin-embedded |
| FIP | Feline infectious peritonitis |
| FISH | Fluorescence *in situ* hybridisation |
| FIV | Feline immunodeficiency virus |
| FLS | Feline leprosy syndrome |
| FN | Female neutered |
| FNA | Fine needle aspirate |
| FTL3L | FMS-like tyrosine kinase 3 ligand |
| GB | Great Britain |
| GC | Guanine-cytosine |
| G-CSF | Granulocyte colony stimulating factor |
| GEAs | Gene expression assays |
| GM-CSF | Granulocyte-monocyte colony stimulating factor |
| GVIF | Generalised variance inflation factor |
| H&E | Haematoxylin and eosin |
| HBHA | Heparin-binding haemagglutinin adhesin |
| HCl | Hydrochloride |
| HIER | Heat induced epitope retrieval |
| HIV | Human immunodeficiency virus |
| hpf | High power field |
| HRM | High resolution melting |
| HRP | Horseradish peroxidase |
| *hsp65* | 65kDa heat shock protein |
| Iba1 | Ionised calcium-binding adaptor protein-1 |

| | |
|---|---|
| IFNγ | Interferon-gamma |
| Ig | immunoglobulin |
| IGRA | Interferon-gamma release assay |
| IHC | immunohistochemistry |
| IL | Interleukin |
| IP | Interferon-gamma inducible protein |
| IS | Insertion sequence |
| ITS | Internal transcribed spacer |
| IU | International units |
| KC | Keratinocytes-derived chemokine |
| LB | Lucia-Bertani |
| LN | Lymph node |
| LR | Likelihood ratio |
| LTBI | Latent tuberculosis infection |
| M | molar |
| M- | Male, unknown neuter status |
| *M.* | *Mycobacterium* |
| MAC | *Mycobacterium avium*-complex |
| MALDI-TOF | Matrix-assisted laser desorption ionisation-time of flight |
| MAP | *Mycobacterium avium* subspecies *paratuberculosis* |
| MAPIA | Multi-antigen print immunoassay |
| Mb | Megabase |
| MBCF | *Mycobacterium bovis* culture filtrate |
| MCP | Monocyte-chemotactic protein |
| MDB | Minimum database |
| MHC | Major histocompatibility complex |
| MIP | Macrophage inflammatory protein |
| MIRU-VNTR | Mycobacterial interspersed repetitive unit-variable number of tandem repeats |
| MN | Male neutered |
| MNGCs | Multinucleated giant cells |
| MR | Magnetic resonance |
| MRCVS | Member of the Royal College of Veterinary Surgeons |
| mRNA | Messenger ribonucleic acid |
| MT | Masson's Trichrome |
| MTBC | *Mycobacterium tuberculosis*-complex |
| NA | Not available |
| NAC | N-acetyl cysteine |
| NaCl | Sodium chloride |
| NC | Negative control |
| NK | Natural Killer |

| | |
|---|---|
| NP | Not performed |
| NPV | Negative predictive value |
| NTM | Non-tuberculous mycobacteria |
| OD | Optical density |
| OR | Odds ratio |
| OTB | Ocular tuberculosis |
| PBMCs | Peripheral blood mononuclear cells |
| PBS | Phosphate buffered saline |
| PC | Positive control |
| PCR | Polymerase chain reaction |
| PDGF-BB | Platelet derived growth factor beta |
| PMF | Peptide mass fingerprint |
| PO | *Per os* |
| PPD | Purified protein derivative |
| PPDA | Purified protein derivative from *Mycobacterium avium* |
| PPDB | Purified protein derivative from *Mycobacterium bovis* |
| PPV | Positive predictive value |
| PVS | Primary veterinary surgeon |
| q24h | Every 24 hours |
| QC | Quality control |
| qPCR | Quantitative polymerase chain reaction |
| ra | Receptor antagonist |
| RANTES | Regulated on activation, normal T-cell expressed and secreted |
| RD | Region of difference |
| RFLP | Restriction fragment length polymorphism |
| RGM | Rapid-growing mycobacteria |
| rIFNγ | Recombinant interferon-gamma |
| RNA | Ribonucleic acid |
| RNAP | Deoxyribonucleic acid-dependent ribonucleic acid-polymerase |
| ROC | Receiver-operator characteristic curve |
| ROI | Region of interest |
| RPE | Retinal pigment epithelium |
| rRNA | Ribosomal ribonucleic acid |
| RT | Room temperature |
| RVN | Registered veterinary nurse |
| RVS | Referring veterinary surgeon |
| *S.* | *Salmonella* |
| SAC | South American camelids |
| SAMe | S-adenosyl-L-methionine |
| SD | Standard deviation |

| | |
|---|---|
| sFas | Soluble Fas |
| sIL-2Rα | Soluble interleukin 2-receptor alpha |
| SLS | Sodium lauryl sulfate |
| SNPs | Single nucleotide polymorphisms |
| SPF | Specific-pathogen-free |
| spp. | Species |
| subsp. | Subspecies |
| TAM | Tetramethylrhodamine |
| TB | Tuberculosis |
| TBS | Tris-buffered saline |
| TBS-T | Tris-buffered saline-Tween® 20 |
| Th | T helper type |
| $T_m$ | Melting temperature |
| TNF | Tumour necrosis factor |
| TST | Tuberculin skin testing |
| UK | United Kingdom |
| USA | United States of America |
| VPU | Veterinary Pathology Unit |
| VEGF | Vascular endothelial growth factor |
| w/v | Weight/volume |
| WGS | Whole genome sequencing |
| ZN | Ziehl-Neelsen |

# Contents

# Chapter 1: Introduction

## 1.1 Feline Mycobacteriosis

Mycobacterial infections in cats have been recognised for over 100 years (Jensen, 1891). However, following practices such as the routine pasteurisation of milk, feline mycobacteriosis has become something of a forgotten disease. However, over the latter half of the 20th and early 21st century there have been increasing reports of feline mycobacterial infections, particularly in Europe, North America, and Australia (Snider *et al.*, 1971, McIntosh, 1982, Gunn-Moore *et al.*, 1996, O'Brien *et al.*, 2017c, Munro *et al.*, 2021). Feline mycobacteriosis appears to be particularly prevalent in Great Britain (GB), where an estimated 1% of all feline tissue biopsy samples submitted to diagnostic histopathology laboratories showed signs consistent with mycobacterial infection (Gunn-Moore *et al.*, 2013). Despite this evident burden of disease in the domestic cat population, our understanding of mycobacterial infections in this species is lacking, especially when compared to the volume of mycobacterial research in other species such as cattle (Buddle *et al.*, 1995, Vordermeier *et al.*, 2001, Denis *et al.*, 2004, Hope *et al.*, 2005). These deficiencies in our knowledge require addressing so that cases of feline mycobacteriosis can be rapidly and accurately diagnosed which would allow for the implementation of appropriate therapy to successfully treat these infections, as well as ensuring steps are taken to safeguard human health.

### 1.1.1 Aetiology

Included within the genus *Mycobacterium* (*M.*) are some of the most successful pathogens of humans, namely *M. tuberculosis*, which causes tuberculosis (TB) (World Health Organization, 2021), and *M. leprae*, the causative agent of leprosy (Cole *et al.*, 2001). Globally, in 2020 there were more than 1.3 million deaths due to TB in humans, with a further 214,000 deaths in those who were also positive for human immunodeficiency virus (HIV) (World Health Organization, 2021). These figures reflect an increase in the number of deaths reported in 2019 and is thought to have been impacted by the sudden acute respiratory syndrome coronavirus-2 pandemic (World Health Organization, 2021). Mycobacterial infections therefore continue to represent a major source of morbidity and mortality in human populations, despite *M. tuberculosis* having been successfully isolated and cultivated in the late 19th century (Barberis *et al.*, 2017). Mycobacteria can also successfully infect and cause disease in animals, ranging from mammals (Broughan *et al.*, 2013), birds (Washko *et al.*, 1998), reptiles (Ebani *et al.*, 2012), amphibians (Rowlatt and Roe, 1966) and fish (Phillips *et al.*, 2017). As with infections in humans, animal mycobacterial infections can cause a significant degree of morbidity and result in economic challenges (Allen *et al.*, 2018, Byrne *et al.*, 2019), as well as pose a risk to human health (Biet *et al.*, 2005).

Mycobacteria are aerobic, curved-to-straight rods approximately 0.2-0.6μm in diameter and 1-10μm in length (Magee and Ward, 2012), with a thick, complex, lipid-rich cell wall containing various mycolic acids (Brennan and Nikaido, 1995). The genome size is variable across the genus, ranging from 3.27-

megabase (Mb) in *M. leprae* (Cole *et al.*, 2001) to 6.99 Mb for *M. smegmatis* (Mohan *et al.*, 2015). They also show wide variety in their doubling time; rapid-growers such as *M. fortuitum* have a doubling time of 8 hours (Hall-Stoodley and Lappin-Scott, 1998), whereas *M. tuberculosis* has an average doubling time of 24 hours (Nair *et al.*, 2009). At the far extreme of this scale is *M. leprae*, with an approximate doubling time of 14 days (Bhat and Prakash, 2012). Mycobacteria also occupy a variety of ecological niches. While some species are considered obligate pathogens (Cole *et al.*, 2001, Allen *et al.*, 2019), other species can be found in ecotypes such as the soil (Barbier *et al.*, 2017, Walsh *et al.*, 2019), water (Falkinham III, 1996, Thomson *et al.*, 2014), and house dust (Torvinen *et al.*, 2010).

The genus consists of over 190 species (Tortoli *et al.*, 2017), and there are multiple ways by which it can be divided. Some of these are based on phenotypic characteristics such as growth rate or pigmentation. Mycobacteria can be divided into rapid- and slow-growers based on the length of time it takes for them to form colonies on solid media, with slow-growers requiring more than seven days, and rapid-growers producing visible colonies between three and seven days (Tortoli, 2003). The division between rapid- and slow-growing species of mycobacteria aligns with the Runyon classification, which divides mycobacteria based on their pigmentation characteristics. The slow-growing mycobacteria are divided into three groups (Runyon I-III), while rapid-growing species are classified as Runyon IV. Runyon I organisms are those that produce pigment when exposed to light and are termed photochromogens, whereas Runyon II organisms, called scotochromogens, produce pigment in both light and dark conditions. Both Runyon III and Runyon IV organisms do not produce pigment, with those in Runyon III termed nonphotochromogens (Runyon, 1959). However, with the advancement of molecular techniques, there is a move towards classifying mycobacteria based on their genomic characteristics and similarities. Broadly, the genus can be divided into two categories:

- The *M. tuberculosis*-complex (MTBC), comprising of those organisms that cause TB in humans and animals
- The non-tuberculous mycobacteria (NTM), consisting of all species that are not members of the MTBC

#### 1.1.1.1 Tuberculosis

Tuberculosis is defined as disease resulting from infection with a member of the MTBC, a group of closely related organisms within the genus *Mycobacterium* (Brites and Gagneaux, 2017, Malone and Gordon, 2017). Members of the MTBC include *M. tuberculosis*, the type species of this genus, *M. africanum*, *M. bovis*, *M. canettii*, *M. caprae*, *M. microti*, *M. mungi*, *M. orygis*, *M. pinnipedii* and *M. suricattae*, and as well as the uncharacterised ‘dassie bacillus’ and ‘chimpanzee bacillus’, and the lab-attenuated organism *M. bovis* bacillus Calmette-Guérin (BCG) (Brites *et al.*, 2018). There is ongoing debate as to the precise classification and nomenclature of these organisms, as well as their inclusion in the MTBC. For example, *M. canettii* is considered a member of the MTBC based on nucleotide

identity, but it has been argued that it is an environmental organism causing opportunistic infection and disease in humans (Koeck *et al.*, 2011). It has also been argued that members of the MTBC should be considered as strains of *M. tuberculosis* and named as such *i.e.*, *M. tuberculosis* variant bovis, given the degree of genetic similarity between these organisms (Riojas *et al.*, 2018). However, most members of the MTBC show high levels of host specificity and occupy particular niches and ecotypes (Smith *et al.*, 2006), other than *M. bovis* which appears capable of infecting and causing disease in a wide range of species (Pollock and Neill, 2002, Corner *et al.*, 2011, Broughan *et al.*, 2013, Miller *et al.*, 2015, Olea-Popelka *et al.*, 2017). Retention of the current naming system is likely to be beneficial to reflect this diversity, as well as for ease of understanding test results by non-specialists.

Tuberculosis in cats is caused by *M. bovis*, most notably associated as the major cause of bovine TB (bTB), and *M. microti*, the vole bacillus (Kipar *et al.*, 2014). These two organisms are the most commonly cultured mycobacterial species of cats, accounting for just over 72% of culture-positive results from submissions to the Animal Health and Veterinary Laboratories Agency (AHVLA) (now Animal and Plant Health Agency [APHA]) (Gunn-Moore *et al.*, 2011a). Cats appear to be inherently resistant to infection with *M. tuberculosis* (Francis, 1958), and previously reported cases of feline TB due to *M. tuberculosis* may have been due to infection with another member of the MTBC, namely *M. microti*. This organism has been highlighted as a major cause of feline TB in GB but previous cases of suspected *M. microti* infection were reported as being attributable to an '*M. microti*-like' organism (Gunn-Moore *et al.*, 1996), which may have resulted in under-reporting of this pathogen. Cases of feline TB due to *M. bovis* have been infrequently reported in other countries (Kaneene *et al.*, 2002, Cerná *et al.*, 2019), notably in those where bTB remains endemic, while cases of *M. microti* infection are being increasingly identified in continental Europe (Rüfenacht *et al.*, 2011, Michelet *et al.*, 2015, Peterhans *et al.*, 2020), likely due to advances in molecular diagnostic techniques for these infections. Recently, a case of TB due to *M. bovis* BCG has been reported (Manou *et al.*, 2021); whether this remains an isolated incident or a potential new threat to cats living in areas undergoing vaccination of wild badger populations remains to be seen.

In GB, cases of TB show clear geographic division between the underlying aetiological agent involved (Gunn-Moore *et al.*, 2011a). Feline *M. bovis* infections are clustered around south-west England and south Wales, areas that are deemed as 'high risk' for bTB (Smith *et al.*, 2009, More *et al.*, 2018). Conversely, infections with *M. microti* are reported in areas that are classified as Officially TB Free such as Scotland, or 'low risk' bTB areas such as the south-east of England (Smith *et al.*, 2009, More *et al.*, 2018). It has been shown that in some regions with a high incidence of feline *M. microti* infections that there is endemic infection of wildlife populations with this pathogen (Burthe *et al.*, 2008, Smith *et al.*, 2009). Techniques such as spoligotyping and genotyping have demonstrated near perfect identity across isolates of *M. bovis* and *M. microti* from cats, cattle and wildlife species (Monies *et al.*, 2000, Smith *et al.*, 2009), which would support the notion that cats do play a role in the greater epidemiology of bTB infections, and that there is interspecies transmission of these pathogens.

### 1.1.1.2 Non-Tuberculous Mycobacteriosis

Whereas the MTBC consists of a reasonably homogenous group of mycobacterial species, the NTM group is much more diverse. Consequently, it has been recently suggested that the NTM should be reclassified among the current genus *Mycobacterium* and four new genera (Gupta *et al.*, 2018); unsurprisingly, this has been rebuked by others (Tortoli *et al.*, 2019).

The most frequently cultured NTM causing disease in cats in GB is *M. avium* (Gunn-Moore *et al.*, 2011a), accounting for 15% of culture-positive submissions. There are four subspecies (subsp.) of *M. avium*: *M. avium* subsp. *avium*, *M. avium* subsp. *hominissuis*, *M. avium* subsp. *silvaticum*, and *M. avium* subsp. *paratuberculosis* (MAP), the causative agent of Johne's Disease in ruminants (Whittington *et al.*, 2019). While it is possible to distinguish between infections with different subspecies of *M. avium* based on the identification of specific genetic markers (Shin *et al.*, 2010) or differences in banding patterns of restriction fragment length polymorphism (RLFP) typing (de Groot *et al.*, 2010), this is not routinely undertaken in cases of feline mycobacteriosis. Therefore, it would be sensible to term these as infections with a *M. avium*-complex (MAC) species, but this terminology can become unclear when considering an expanded definition of the MAC. At its broadest, the MAC can be thought of as comprising at least 12 different species (van Ingen *et al.*, 2018), including *M. intracellulare* (Deykin *et al.*, 1996, Davies *et al.*, 2006) and *M. lepraemurium* (O'Brien *et al.*, 2017c).

Feline leprosy syndrome (FLS) has been historically attributed to infection with *M. lepraemurium* (McIntosh, 1982, Hughes *et al.*, 1997). This term was applied to cases of cutaneous and subcutaneous mycobacteriosis where organisms could not be cultured on media and cases have been reported globally (McIntosh, 1982, Malik *et al.*, 2002, Laprie *et al.*, 2013). Advances in molecular techniques have revealed many other causative agents of FLS other than *M. lepraemurium*, including *M. visibile* (Appleyard and Clark, 2002), *Candidatus* (*C.*) 'M. tarwinense' (O'Brien *et al.*, 2017b) and *C.* 'M. lepraefelis' (O'Brien *et al.*, 2017a), and it has been shown that the clinicohistopathological features of FLS can vary greatly between individuals (Malik *et al.*, 2002). Furthermore, classification of *M. lepraemurium* as a member of the MAC indicates crossover between disease due to MAC infection and FLS, and cases diagnosed as FLS on the basis of clinical signs have been shown to arise from infection with cultivable NTM such as *M. kansasii* (Foley *et al.*, 2004) and even members of the MTBC (Laprie *et al.*, 2013). Therefore, the term FLS may be considered somewhat obsolete as a distinct disease presentation.

Many other NTM are responsible for causing disease in cats. This includes slow-growing organisms such as *M. malmoense* (Gunn-Moore *et al.*, 2011a, Hetzel *et al.*, 2012, Karkamo *et al.*, 2016, Pekkarinen *et al.*, 2018) and *M. kansasii* (Lee *et al.*, 2017, Černá *et al.*, 2020), both of which are potential human pathogens (Engel *et al.*, 1981, Penny *et al.*, 1982, Doig *et al.*, 2002), as well as rapid-growers including *M. fortuitum* (Malik *et al.*, 2000, Jang and Hirsh, 2002, Couto and Artacho, 2007, Carlotti *et al.*, 2009, Gunn-Moore *et al.*, 2011a) and *M. smegmatis* (Malik *et al.*, 1994, Alander-Damsten *et al.*, 2003, Munro *et al.*, 2021). Other species of NTM that have been reported in the literature as causing

feline mycobacteriosis include, but are not limited to, *M. abscessus-chelonae* (Jang and Hirsh, 2002, Munro *et al.*, 2021), *M. alvei* (Beccati *et al.*, 2007), *M. branderi/shimoidei* (Pekkarinen *et al.*, 2018), *M. celatum* (Gunn-Moore *et al.*, 2011a), *M. chitae* (Hughes *et al.*, 1997)*, M. flavescens* (Jang and Hirsh, 2002), *M. genavense* (Hughes *et al.*, 1999), *M. heckeshornense* (Elze *et al.*, 2013), *M. nebraskense* (Niederhäuser *et al.*, 2018), *M. porcinum* (Cox and Udenberg, 2020), *M. simiae* (Dietrich *et al.*, 2003), *M. setense* (Apostolopoulos *et al.*, 2021), *M. terrae*-complex (Henderson *et al.*, 2003), *M. thermoresistibile* (Foster *et al.*, 1999, Vishkautsan *et al.*, 2016, Munro *et al.*, 2021), *M. ulcerans* (Elsner *et al.*, 2008), and *M. xenopi* (Meeks *et al.*, 2008). This demonstrates the vast range of species of mycobacteria capable of causing disease in the cat, and further highlights the difficulty in obtaining an accurate diagnosis to facilitate the appropriate treatment required for these infections.

### 1.1.2 Risk Factors and Routes of Transmission

Cases of mycobacteriosis have been reported in cats of all ages (Munro *et al.*, 2021); while the median age of cats with *M. bovis* infections in GB is younger than that of those infected with *M. microti* (3 years *vs.* 8 years, respectively) (Gunn-Moore *et al.*, 2011a), there can be a substantial degree of overlap. Regarding cases of NTM disease, infections with *M lepraemurium* tend to be reported in younger cats (median age 2 years) (O'Brien *et al.*, 2017c), whereas *C.* 'M. lepraefelis' infections are more likely in older animals (median age 9.5 years) (O'Brien *et al.*, 2017a). This assertion had been documented previously, with *M. lepraemurium* identified as causing disease in younger cats compared to an unidentified mycobacterial species infecting older cats (Malik *et al.*, 2002). There is a tendency towards infection being reported in more male cats than females (Gunn-Moore *et al.*, 2011a, O'Brien *et al.*, 2017a, O'Brien *et al.*, 2017c), although this male-bias is not reported in all studies (McIntosh, 1982, Smith *et al.*, 2009, Major *et al.*, 2016, O'Brien *et al.*, 2017b). Breed dispositions have been suggested for *M. avium* infections, notably in Abyssinian (Baral *et al.*, 2006) and Siamese cats (Jordan *et al.*, 1994); whether there are similarities with reported heritable immunodeficiencies that predispose some breeds of dog to *M. avium* infection (Ghielmetti and Giger, 2020) has not been elucidated as of yet.

Pertaining to lifestyle, cases of feline TB had been associated with consumption of raw milk from tuberculous cows (Snider *et al.*, 1971), but routine pasteurisation has all but eliminated this risk. However, known hunting behaviour by outdoor cats is a routine feature of many current cases of feline mycobacterial infection (Gunn-Moore *et al.*, 1996, Gunn-Moore *et al.*, 2011a, O'Brien *et al.*, 2017a, O'Brien *et al.*, 2017b, O'Brien *et al.*, 2017c, Munro *et al.*, 2021). This, combined with the distribution of lesions (see *1.1.3 Clinical Signs*) and the shared genotypes of *M. bovis* and *M. microti* isolates in cases of TB in cats and wildlife species (Smith *et al.*, 2009) would suggest that hunting of infected rodents is a major route of infection for cats with mycobacteria. Alternatively, soil contamination of wounds, particularly of the ventral abdomen, may also be a route of transmission, especially with lipophilic rapid-growing NTM. This may suggest obesity as a risk factor for infections with this subset of mycobacteria (Malik *et al.*, 2000). A recent outbreak of *M. bovis* infection in predominantly indoor, pedigree cats

across Scotland and low risk bTB areas of England identified consumption of a particular brand and flavour of a commercial raw food diet as the sole consistent factor in these cases (O'Halloran *et al.*, 2019, O'Halloran *et al.*, 2020). Consumption of suspected contaminated food resulting in *M. bovis* infection in a housed research cat has also been documented (Isaac *et al.*, 1983). Therefore, raw feeding should be considered another potential risk factor for cases of feline mycobacteriosis and a thorough dietary history should be obtained in all cases where mycobacterial infection is suspected (Mitchell *et al.*, 2021a).

Classical causes of immunosuppression in cats, namely infection with the retroviruses feline leukaemia virus (FeLV) or feline immunodeficiency virus (FIV), are less frequently identified as a risk factor for mycobacterial infection compared to HIV-TB in humans, for example. Neither retrovirus is typically associated with cases of feline mycobacteriosis in GB (Gunn-Moore *et al.*, 2011a), although in some multicat households where feline TB has been documented the number of FIV-positive cats was higher than expected from the population (Monies *et al.*, 2000). There have also been reports of FIV-NTM co-infection (Hughes *et al.*, 1999, Paharsingh *et al.*, 2020) (see *1.1.4.1.3 Retrovirus Testing*). Other potential causes of immunosuppression have been identified in singular case reports, such as idiopathic cluster of differentiation (CD)4+ lymphopenia (Meeks *et al.*, 2008), ciclosporin treatment following renal transplantation (Griffin *et al.*, 2003), lymphoma (potentially secondary to FIV infection) (Elze *et al.*, 2013), renal interstitial nephritis (Pekkarinen *et al.*, 2018) and chronic kidney disease with secondary *Cryptococcus neoformans/gattii* complex infection (Graham *et al.*, 2011). Treatment with ciclosporin in cases of feline atopic dermatitis resulting in secondary mycobacterial infection has been identified in a small number of cats (J. Mitchell, unpublished data).

Living in a multicat household has not been identified as a risk factor for cases of mycobacteriosis, and there have been very few reports of cat-to-cat or human-to-cat transmission of mycobacteria. Infection of other cats is thought to arise from inhalation of mycobacteria present in discharging sinus tracts (Isaac *et al.*, 1983), or may occur from ingestion of organisms through allogrooming or contamination of wounds in cases of close contact (Murray *et al.*, 2015, Černá *et al.*, 2020). It can also be difficult to discern whether multiple cases of mycobacteriosis within the same household arise from cats hunting the same infected wildlife population or another environmental source of infection, or whether infection has been transmitted from individual to another (Monies *et al.*, 2000, Murray *et al.*, 2015). As with cases of cat-to-cat transmission, human-to-cat transmission is thought to occur when there is close contact between individuals and signs of active disease in the owner (Ramdas *et al.*, 2015).

Nosocomial infections are incredibly rare; however, it poses a real risk for cases of mycobacteriosis given that cats with discharging lesions may not be handled with appropriate hygiene measures (see *1.1.3 Clinical Signs*), resulting in environmental contamination and subsequent infection of others if cleaning practices are insufficient. One small outbreak of *M. bovis* nosocomial infection has been described (Murray *et al.*, 2015), in addition to another case of suspected nosocomial infection as part of a localised cluster of *M. bovis* infections (Roberts *et al.*, 2014).

The role of vectors in the transmission of mycobacterial infections has been overlooked. Recently, mycobacterial deoxyribonucleic acid (DNA) was isolated from fleas (Zurita *et al.*, 2021) and ticks (Khoo *et al.*, 2016), but the exact species of mycobacteria could not be identified. It is likely the impact of ectoparasites on mycobacterial transmission is minimal, but it warrants further investigation.

### 1.1.3 Clinical Signs

Cases of feline mycobacteriosis may present with non-specific signs of ill health such as hyporexia/anorexia, weight loss and lethargy (O'Halloran *et al.*, 2020), but there may be lesions identified by the owner resulting in presentation to the veterinary clinic. Cutaneous lesions are the most common finding, reported in 74% of cases of mycobacteriosis in GB (Gunn-Moore *et al.*, 2011a) (Figure 1.1); these are typically located in 'fight and bite' sites *i.e.*, the head, distal extremities, perineum, and tail base, but can be distributed all over the body. Lesions are often raised and alopecic, and there may be additional findings such as ulceration and/or discharging sinus tracts (Gunn-Moore *et al.*, 2011a). Cutaneous lesions are seen in cases of MTBC and NTM infection (Gunn-Moore *et al.*, 1996, Malik *et al.*, 2013), hence the clinical presentation cannot be used to determine which species or group of mycobacteria is responsible for causing disease (Figure 1.2). However, one presentation of disease that appears to be specific to infection with one group of mycobacteria is that of panniculitis. This is reported in cases of infection with rapid-growing NTM (Malik *et al.*, 1994, Youssef *et al.*, 2002, Alander-Damsten *et al.*, 2003, Beccati *et al.*, 2007, Vishkautsan *et al.*, 2016, Cox and Udenberg, 2020, Apostolopoulos *et al.*, 2021, Munro *et al.*, 2021), presenting as multiple punctate draining tracts and thickening of the subcutaneous and cutaneous tissues (Gunn-Moore, 2014), particularly of the ventral abdomen and other tissues with a high concentration of lipids (Figure 1.3). These lesions can be extensive and painful, resulting in pyrexia and a reluctance of the cat to move.

Lymphadenopathy is reported in nearly half of all cases of feline mycobacteriosis in GB (Gunn-Moore *et al.*, 2011a); this may involve the lymph nodes local to the cutaneous lesion or reflect generalised lymph node involvement. In some cases, there is no discernible cutaneous involvement in the face of lymph node lesions; this is termed an 'incomplete primary complex' (Gunn-Moore *et al.*, 1996) (Figure 1.4).

Pulmonic involvement is thought to arise following haematogenous dissemination of mycobacteria from the primary lesion to the lungs (Bennett *et al.*, 2011, Ganbat *et al.*, 2016), although in rare cases there may be inhalation of mycobacteria resulting in primary respiratory disease (O'Halloran *et al.*, 2019). In cases with secondary pulmonic involvement, disease is interstitial and progressive, eventually becoming bronchial (see *1.1.4.2 Diagnostic Imaging*). This presents clinically as dyspnoea and an increase in the resting respiratory rate (Albuquerque *et al.*, 2021); a soft, productive cough is rare but may be identified in severe cases (O'Halloran *et al.*, 2020).

**Figure 1.1:** A focal, non-ulcerated granuloma on the upper lip margin (black arrow) of a two-year-old neutered female (FN) domestic shorthair (DSH) cat diagnosed with *M. bovis* infection on polymerase chain reaction (PCR) testing. Image courtesy of Rebecca Sheppard, Member of the Royal College of Veterinary Surgeons (MRCVS).

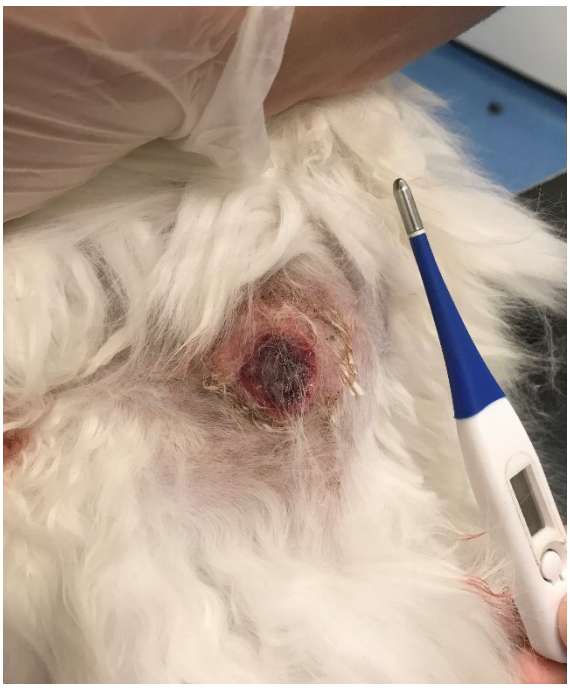

**Figure 1.2:** A focal, well circumscribed, raised, alopecic, ulcerated lesion in the pectoral region of a seven-year-old neutered male (MN) domestic longhair (DLH) cat diagnosed with *M. avium* infection on a fine needle aspirate (FNA) biopsy of the lesion with subsequent PCR. Note the wearing of gloves (top left). Image courtesy of Laura Vico MRCVS and Elise Cordell, Registered Veterinary Nurse (RVN).

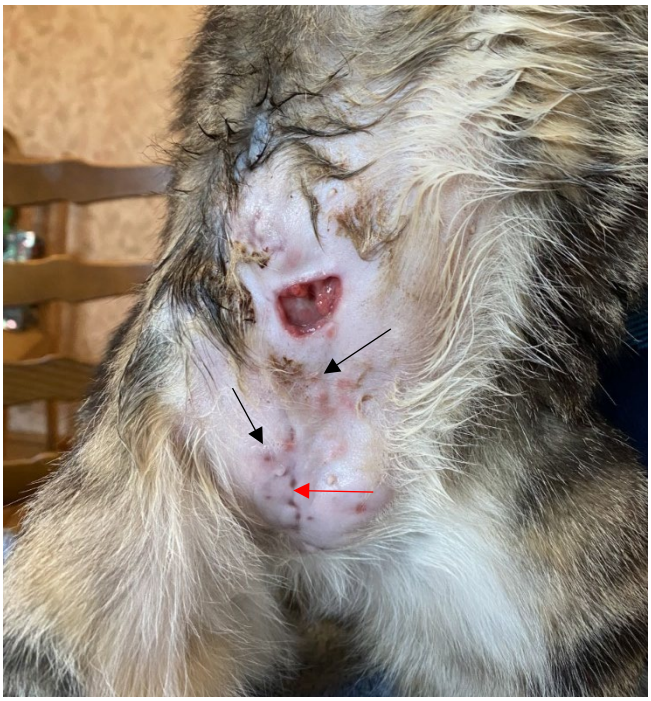

**Figure 1.3:** Severe panniculitis and ulceration of the ventral abdomen in an 11-year-old MN DSH cat, diagnosed with *M. smegmatis* on PCR testing on a FNA of the affected tissue. In addition to the site of ulceration there are multiple punctate lesions caudally (black arrows) and the skin was grossly thickened. Note the surgical scar towards the caudal aspect of the affected tissue (red arrow); this cat had previously suffered a traumatic laceration and a glass foreign body was recovered from the site of injury. This likely provided a portal of entry for the mycobacteria. Interferon-gamma (IFNγ) release assay (IGRA) testing gave an equivalent response to avian and bovine tuberculin. Image courtesy of the owner and Alice Turner MRCVS.

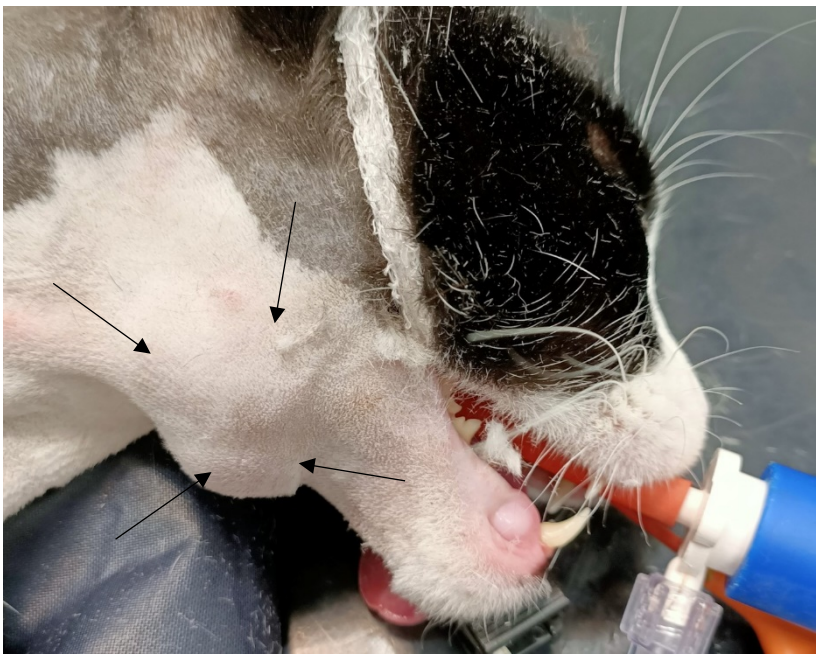

**Figure 1.4:** Marked bilateral mandibular lymphadenopathy (outlined with black arrows) in a three-year-old MN DSH cat; no primary cutaneous lesion was identified. A diagnosis of MTBC infection was made on PCR, and *M. microti* was suspected as this cat lived in a low-risk area for bTB and had not been fed the commercial raw food diet associated with an outbreak of *M. bovis* in cats. He was treated for six months with rifampicin, azithromycin and pradofloxacin; six months after treatment was stopped, he was reported to be clinically healthy with resolution of the lymphadenopathy. Image courtesy of Ruth Wadsworth MRCVS.

Abdominal lesions are less common, following practices such as the routine pasteurisation of milk (Snider *et al.*, 1971), but in a recent outbreak of *M. bovis* infection associated with a commercial raw food diet almost half (49%) of the cats involved had palpable abdominal lesions (O'Halloran *et al.*, 2020). When abdominal lesions are reported, this typically involves thickening of the intestines and multicentric lymphadenopathy, especially of the mesenteric lymph nodes; other abdominal lesions include hepatosplenomegaly and abdominal masses (Snider *et al.*, 1971, Baral *et al.*, 2006, Fitzgerald *et al.*, 2016, Cerná *et al.*, 2019, O'Halloran *et al.*, 2019, O'Halloran *et al.*, 2020). This can result in signs such as vomiting, diarrhoea and weight loss.

Less commonly identified clinical signs include lameness and joint swelling (Lalor *et al.*, 2017, Munro *et al.*, 2021), acute onset blindness, retinal detachment and signs of uveitis (Formston, 1994, Stavinohova *et al.*, 2019), the development of effusions (pericardial, pleural or peritoneal) (O'Halloran *et al.*, 2020, Paharsingh *et al.*, 2020), and renal lesions (Monies *et al.*, 2000). Signs attributable to lesions in the central nervous system (CNS) have also been identified (Madarame *et al.*, 2017), including hypermetria (Blauvelt *et al.*, 2001), and ataxia (Baral *et al.*, 2006).

### 1.1.4 Diagnostic Testing

Given the non-specific clinical signs, and the requirement to obtain a species- or group-level diagnosis to inform treatment protocols, prognosis and zoonotic risk, diagnostic testing needs to be performed. However, accurately diagnosing mycobacterial infections can be lengthy, expensive, and a positive result may not be attained despite testing with multiple modalities. Initial diagnostic investigations may help to rule out more likely causes of the clinical signs or highlight additional findings that increase the index of suspicion of mycobacterial disease.

#### 1.1.4.1 Laboratory Testing

As mycobacterial disease may not be high on the list of differential diagnoses when a clinician is initially presented with a case of mycobacteriosis, a set of non-specific tests may be performed to obtain the ‘minimum database’ (MDB) as an indicator of general health and wellbeing. The MDB for cats will vary depending on clinician preference, practice capability and the age of the animal, but is likely to include a combination of haematology, serum biochemistry, urinalysis, thoracic and abdominal imaging, retrovirus testing and serum thyroxine testing (Kipperman, 2014).

#### 1.1.4.1.1 Haematology and Serum Biochemistry

Haematological testing may reveal abnormalities in both red and white blood cell parameters, however, these changes are non-specific for mycobacterial disease. A non-regenerative anaemia, likely anaemia of inflammatory disease (Chikazawa and Dunning, 2016) is infrequently identified, and this could be associated with more severe presentations of disease such as disseminated infection (Baral *et al.*, 2006, Cerná *et al.*, 2019, O'Halloran *et al.*, 2019, O'Halloran *et al.*, 2020). Leucocytosis may also be reported, driven by a neutrophilia (O'Halloran *et al.*, 2019, O'Halloran *et al.*, 2020), and in some cases there may be band neutrophils, indicating a left shift and suggesting a more severe inflammatory process (Baral *et al.*, 2006). Where there is overwhelming infection the immune system may collapse, resulting in neutropenia with evidence of toxic change (Cerná *et al.*, 2019). It has been shown that the median monocyte to lymphocyte ratio is higher in humans with active TB compared to healthy controls, and monitoring this may provide a means for assessing the response to treatment (Wang *et al.*, 2015); whether this is also true in cats has not been investigated.

Changes on serum biochemistry are similarly non-specific. The changes most commonly identified include hypercalcaemia (see *1.1.4.1.2 Calcium and Vitamin D*), increased liver enzymes and alterations in protein concentrations, reflective of an acute phase response *i.e.*, decreased albumin and/or increased globulin, which may result in an overall hyperproteinaemia (Baral *et al.*, 2006, O'Halloran *et al.*, 2020). Other changes such as an increase in renal parameters (O'Halloran *et al.*, 2019, Černá *et al.*, 2020) may be reported, but the significance of these findings may not be necessarily attributable to mycobacterial infection.

#### 1.1.4.1.2 Calcium and Vitamin D

A recognised clinicopathological feature of granulomatous inflammatory disorders is that of hypercalcaemia (Sharma, 2000). Calcium is an essential mineral for many biological processes, and it is under tight homeostatic control mechanisms mediated by parathyroid hormone, calcitonin and vitamin D (Moe, 2016), the active form of which is 1,25-dihydroxyvitamin D (1,25-$(OH)_2$D). Hypercalcaemia associated with granulomatous inflammation occurs due to the increased conversion of inactive 25-hydroxyvitamin D (25(OH)D) to active 1,25-$(OH)_2$D, mediated by 1α-hydroxylase; the gene for this enzyme is expressed by macrophages (Monkawa *et al.*, 2000). This increase in 1α-hydroxylase results in the conversion from 25(OH)D to 1,25-$(OH)_2$D, which acts on the vitamin D receptor and results in an increase in absorption of calcium across the intestine, increased renal reabsorption, and release of calcium from bone, resulting in hypercalcaemia (Fleet, 2017).

Hypercalcaemia in association with mycobacterial disease in cats has been reported infrequently, in both cases of MTBC and NTM infections (Malik *et al.*, 2002, Baral *et al.*, 2006, Gunn-Moore *et al.*, 2011a, Albuquerque *et al.*, 2021). In GB, hypercalcaemia was identified in 23% of cats with

mycobacterial disease (Gunn-Moore *et al.*, 2011a); however, the overall number of cats that had calcium concentration measured was small, so this figure may over- or underestimate the true prevalence of mycobacteriosis-associated hypercalcaemia in cats. Of those cases where hypercalcaemia was reported disease was often disseminated, including respiratory involvement (Baral *et al.*, 2006, Gunn-Moore *et al.*, 2011a), which may suggest that hypercalcaemia is associated with more severe presentations of disease. Therefore, the identification of hypercalcaemia should make the clinician consider a granulomatous inflammatory process, and by extension the causes of this including mycobacterial infection, and it may provide a parameter by which the response to treatment can be monitored (Albuquerque *et al.*, 2021); however, it is neither sensitive nor specific for mycobacterial infection in the cat.

Given that hypercalcaemia may be identified in cats with mycobacterial infection, it would be logical that these cats would also have increased levels of vitamin D; however, this may not be the case. In contrast, it has been shown that cats with mycobacterial disease had lower serum concentration of 25(OH)D compared to healthy control cats (Lalor *et al.*, 2012); concurrent measurement of calcium was not performed to demonstrate whether these cats had low serum vitamin D in the face of hypercalcaemia. This discrepancy between low vitamin D and hypercalcaemia may arise from the fact that the storage form of vitamin D was measured (*i.e.,* 25(OH)D) rather than active $1,25(OH)_2D$ (Brighenti *et al.*, 2018); therefore, low concentrations of 25(OH)D may reflect increased extra-renal conversion of 25(OH)D to $1,25(OH)_2D$ driven by macrophage-encoded 1α hydroxylase. It was also shown that serum concentrations of 25(OH)D were decreased in hospitalised, sick cats without mycobacterial infection (Lalor *et al.*, 2012), and there was no difference in serum 25(OH)D concentration between cats with systemic or cutaneous mycobacteriosis. Additionally, low serum 25(OH)D concentrations have been reported in cats with FIV infections (Titmarsh *et al.*, 2015b), inflammatory bowel disease and intestinal small cell lymphoma (Lalor *et al.*, 2014) and decreased serum 25(OH)D concentrations have been reported as a predictor for 30-day mortality (Titmarsh *et al.*, 2015a). Consequently, assessment of vitamin D in cats may simply reflect a state of ill health and not be necessarily informative in the diagnostic investigation of cases of mycobacteriosis.

#### 1.1.4.1.3 Retrovirus Testing

Testing for the immunosuppressive retroviruses FeLV and FIV is recommended if cats show clinical signs of illness (Little *et al.*, 2020). A recent study of cats in two shelters in the United Kingdom (UK) found an overall prevalence of 9.5% for anti-FIV antibodies and 2.3% for FeLV antigen (Stavisky *et al.*, 2017); recent figures for the prevalence of either infection in the domestic cat population in the UK at large are not readily available, but it is advised to test for both infections when faced with a case of suspected mycobacteriosis.

One reason for doing so is the fact that infection with HIV has been identified as risk factor for humans developing TB, and HIV-TB coinfection is increasingly recognised (World Health Organization, 2021). Feline FIV infections have been used as a model for cases of human infection with HIV (Siebelink *et al.*, 1990), given the similarities between these two viruses (Pedersen *et al.*, 1987), therefore given the burden of HIV-TB co-infection it could be hypothesised that FIV infection would increase the risk of mycobacterial disease in cats. Unlike in humans, there is no clear consensus that feline retroviral infections are a risk factor for developing mycobacterial disease. Only 2.8% of cats in GB diagnosed with mycobacteriosis were FIV-positive, and no FeLV positive cats were identified (Gunn-Moore *et al.*, 2011a). Other studies have reported higher prevalence of FIV-mycobacterial co-infections in Australia and New Zealand, ranging from 11-43% (Malik *et al.*, 2002, O'Brien *et al.*, 2017a, O'Brien *et al.*, 2017b, O'Brien *et al.*, 2017c), and even higher in a study in cats with NTM infections in the United States of America (USA) (Munro *et al.*, 2021). However, it should be noted that the background seroprevalence of FIV in healthy cats in Australia is higher compared to the UK (Hosie *et al.*, 1989, Westman *et al.*, 2016), which may partially explain this difference of rates of FIV-mycobacterial co-infection. Also, the number of cats for which results of retrovirus testing were available in the aforementioned studies on mycobacteriosis was much smaller than the reported value from cats in GB; co-infection with FeLV was not documented in any of these cases. Individual case reports have also shown FIV-mycobacteria co-infections (Hughes *et al.*, 1999, Monies *et al.*, 2000, Elze *et al.*, 2013, Paharsingh *et al.*, 2020). However, depending on the stage of infection, a diagnosis of FIV positivity may not equate to active immunosuppression of that cat at that moment in time (Little *et al.*, 2020). While FeLV infection has not been diagnosed in many cases of mycobacterial infection in cats, it was diagnosed by immunofluorescence of bone marrow aspirates in one cat with disseminated *M. avium* infection (Latimer *et al.*, 1997).

Another concern surrounding retroviral co-infection is the impact on cell numbers, in particular CD4+ cells, which may complicate the diagnosis of mycobacterial infection using tests of cell-mediated immunity (CMI) such as the IGRA. Infection with HIV results in a decrease in the number of CD4+ cells (Douek *et al.*, 2002), which are potent secretors of IFNγ, and it has been identified that low CD4+ counts can make diagnosing mycobacterial infections in humans more difficult (Geldmacher *et al.*, 2008, Aabye *et al.*, 2009). As the number of cats with FIV-mycobacteria co-infection is small it has not been possible to elucidate whether FIV infection complicates the diagnoses of mycobacterial disease using IGRA, but it may be recommended to pursue alternative testing in such cases, such as PCR, to directly identify the pathogen rather than detect an immune response in an immunocompromised individual.

### 1.1.4.2 Diagnostic Imaging

At some point in the investigative process, it is likely that some form of imaging will be performed. This may be performed at the point of initial presentation, at a subsequent revisit following a lack of response to empirical treatment, or after a diagnosis has been made in order to stage the extent of disease which

will inform treatment protocols (O'Halloran and Gunn-Moore, 2017). Imaging can also be useful for monitoring the response to treatment (see *1.1.5 Treatment and Monitoring the Response to Treatment*).

#### 1.1.4.2.1 Radiography

Radiography is the most used imaging modality in cases of feline mycobacteriosis given its availability in general practice, and it allows for the assessment of disease dissemination and whether lesions are locally destructive. Thoracic radiography is recommended in all cases of feline mycobacteriosis (O'Halloran and Gunn-Moore, 2017), as changes are frequently present (Bennett *et al.*, 2011, Gunn-Moore *et al.*, 2011a, Roberts *et al.*, 2014) and in some cases there may be evidence of pulmonic involvement in the absence of respiratory signs (Gunn-Moore *et al.*, 1996, Baral *et al.*, 2006). A bronchial pattern is seen in almost 50% of cases, but a mix of patterns can be identified including alveolar, nodular interstitial and unstructured interstitial disease (Bennett *et al.*, 2011) (Figure 1.5). However, a mixed broncho-interstitial pattern is not pathognomonic for mycobacterial disease in cats; other differentials to consider include toxoplasmosis, lungworm infection, neoplasia, feline asthma, chronic bronchitis, and idiopathic pulmonic fibrosis. In some cases, there may be mycobacterial infection with pre-existing changes due to feline asthma, or co-infection with *Mycoplasma*. Cavitating lesions are rare (O'Halloran *et al.*, 2020) and are thought to arise from inhalation of mycobacteria resulting in primary respiratory disease rather than the interstitial pattern attributed to haematogenous dissemination of mycobacteria to the lungs. Pleural and pericardial effusions have also been reported (Gunn-Moore, 2014) (Figure 1.6). Perihilar lymphadenopathy may be seen (Bennett *et al.*, 2011), reflecting either a reactive process secondary to pulmonic changes or infection of the lymph node itself, while sternal lymphadenopathy is less common.

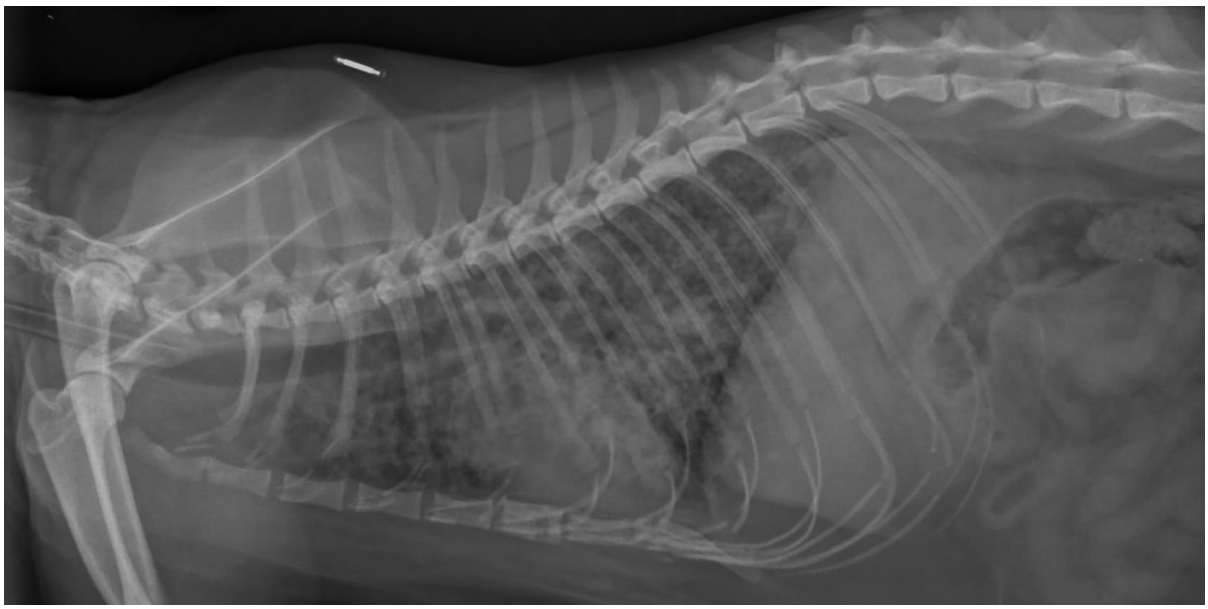

**Figure 1.5:** Right lateral thoracic radiograph of a nine-year-old FN DLH cat showing a marked generalised nodular pattern affecting the lung fields, with loss of detail of the cardiac silhouette. A diagnosis of MTBC infection was made on IGRA; given the cat had not lived outside of Scotland, and had not been fed a raw food diet, a diagnosis of *M. microti* was considered most likely. Image courtesy of Claire Webster MRCVS.

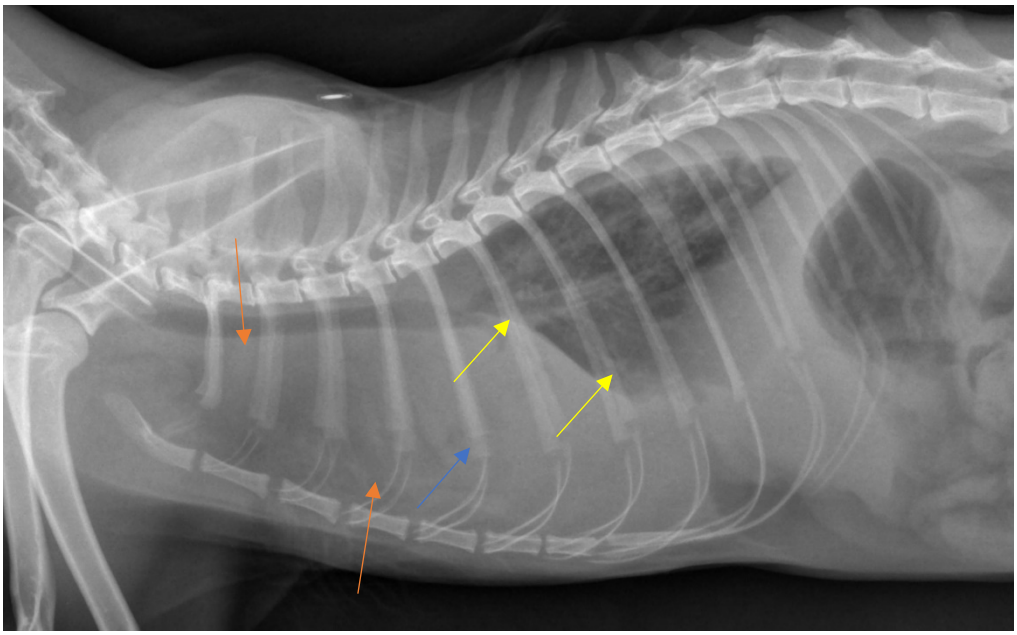

**Figure 1.6:** Left lateral thoracic radiograph of a three-year-old FN Russian Blue cat. There is a soft tissue opacity occupying the cranioventral portion of the thorax (orange arrows), obscuring the cardiac silhouette (blue arrow), and dorsal displacement of the lung fields (yellow arrows). This is consistent with a pleural effusion; thoracocentesis and cytological analysis revealed this to be a chylothorax. The lung fields also show a mild, patchy interstitial pattern. Abdominal ultrasonography identified an enlarged mesenteric lymph node and thickening of the ileum (not shown here). Both IGRA and PCR testing on formalin-fixed paraffin-embedded (FFPE) tissue of the abdominal lesions were negative, despite positive Ziehl-Neelsen (ZN) staining for acid-fast bacilli on the FFPE biopsy. This cat lived indoors and had been historically fed the commercial raw food diet associated with an outbreak of *M. bovis* in cats. Results of antibody testing (see Figure 6.5 A) suggested infection with a MTBC pathogen. Image courtesy of Chris Wilson MRCVS.

Radiography can also be used to assess for the presence of abdominal lesions, but other modalities may be preferable for a more thorough evaluation (Bennett *et al.*, 2011, O'Halloran *et al.*, 2020). Significant organomegaly can be identified on abdominal radiography (Baral *et al.*, 2006, Bennett *et al.*, 2011, O'Halloran *et al.*, 2020), as well as accumulation of fluid within the peritoneal cavity, abdominal masses and mineralisation of lymph nodes (Gunn-Moore, 2014). Other uses of radiography include determining whether there are bone or joint changes present. Bone lesions are typically osteolytic, but osteoproliferative changes may also be identified (Gunn-Moore *et al.*, 2010, Bennett *et al.*, 2011, Lalor *et al.*, 2017, Cerná *et al.*, 2019).

### 1.1.4.2.2 Ultrasonography

Ultrasonography is particularly useful for assessment of the abdominal viscera and may identify lesions missed on radiography, such as abdominal lymphadenomegaly (Sieber-Ruckstuhl *et al.*, 2007, O'Halloran *et al.*, 2020), but it can also be used for characterising thoracic lesions (Foster *et al.*, 1999). Ultrasound can provide better visualisation of the abdomen than radiography, highlighting the location of abdominal lesions and identifying pockets of free fluid (O'Halloran *et al.*, 2019). It can also be used to guide sampling of lesions for cytology (Foster *et al.*, 1999) (see *1.1.4.3.1.1 Cytology*).

#### 1.1.4.2.3 Advanced Imaging: Computed Tomography and Magnetic Resonance Imaging

Computed tomography (CT) is increasingly available in general practice veterinary medicine, and has been used in the investigation of cases of feline mycobacteriosis (Major *et al.*, 2016, Lalor *et al.*, 2017, Černá *et al.*, 2020, O'Halloran *et al.*, 2020, Albuquerque *et al.*, 2021). One advantage of CT over conventional radiography is the increased sensitivity of this modality, allowing for the identification of smaller lesions that would be otherwise missed. Thoracic abnormalities are frequently identified, typified by a structured interstitial pattern (Major *et al.*, 2016), although a variety and combinations of pulmonic patterns are observed. Enlargement of the sternal, cranial mediastinal and/or tracheobronchial lymph nodes is also a common finding on thoracic CT and use of this modality can be highly beneficial for identifying abdominal lesions (Major *et al.*, 2016). Computed tomography has also been used for characterising bone and joint lesions (Major *et al.*, 2016, Lalor *et al.*, 2017, Černá *et al.*, 2020), with cortical lytic lesions taken to indicate sites of primary inoculation whereas periarticular and articular lesions likely represent sites of haematogenous dissemination of mycobacteria.

The use of magnetic resonance (MR) imaging for cases of feline mycobacteriosis has not been fully explored, possibly as it is mostly used to assess the central nervous system (Labruyère and Schwarz, 2013), and neurological signs are rarely reported in cases of feline mycobacterial disease (Blauvelt *et al.*, 2001, Baral *et al.*, 2006). However, MR imaging can also be of benefit for assessing articular structures (Labruyère and Schwarz, 2013), but the relative lack of availability of MR imaging to CT may preclude its use for this purpose in general practice. Currently, the MR findings of feline mycobacterial lesions have only been reported in one cat (Černá *et al.*, 2020), where a neurological component was suspected; MR imaging identified multiple expansive soft tissue lesions resulting in osteolysis, invasion of the surrounding musculature and enlargement of the local lymph nodes. These findings were corroborated with CT.

### 1.1.4.3 Specific Testing

Following from the initial diagnostic investigations described above, specific testing for mycobacteriosis needs to be employed to provide a definitive diagnosis and to inform how best to manage such a case. Broadly, there are two main categories of tests for mycobacteriosis: direct detection *i.e.*, identification of the pathogen, and indirect detection *i.e.*, assessment of the immune response to mycobacterial infection. Some tests available for the diagnosis of mycobacteriosis may be applicable for multiple host-species, namely those of pathogen detection, but not all diagnostics are routinely used for feline infections.

#### 1.1.4.3.1 Pathology

Pathology is incredibly useful for the diagnosis of mycobacterial infections and is often the first indication to the clinician that they are dealing with a case of feline mycobacteriosis. This is either achieved through cytology or histopathology, with special staining.

##### 1.1.4.3.1.1 Cytology

An FNA of lesions can be performed to obtain samples for cytological evaluation and provides a non-invasive method of obtaining a sample which may help rule out other causes for suspicious lesions such as lymphoma (Rivière *et al.*, 2011, O'Halloran and Gunn-Moore, 2017). However, care must be taken to preserve cellular and nuclear integrity, and some firmer lesions may not readily exfoliate on FNA sampling (Powell, 2018), both of which can result in a non-diagnostic sample. Representative samples that have undergone Romanowsky staining will show evidence of macrophages and possibly neutrophils (Baral *et al.*, 2006, Malik *et al.*, 2013), although in some cases cytological examination may simply identify non-specific inflammation. A diagnosis of mycobacteriosis can be made on cytology alone if 'negative-staining ghost bacilli' are identified within the cytoplasm of macrophages (Figure 1.7), or more rarely multinucleated giant cells (MNGCs) (Malik *et al.*, 2002, Lamagna *et al.*, 2009), and subsequent staining with acid-fast stains such as ZN, or a modification of this technique such as Fite-Faraco (FF), reveal the intracellular organisms to be acid-fast bacilli (AFB) (Černá *et al.*, 2020). However, the number of mycobacteria present within a cytological sample can be variable and organisms may be rare or absent. Macrophages laden with abundant intracellular AFB may be termed 'virchowcytes' (Malik *et al.*, 2002) and it has been suggested that cases of FLS are characterised with abundant AFB on cytological examination (Malik *et al.*, 2013).

While cytological examination in cases of mycobacteriosis is often of cutaneous, subcutaneous, or lymph node lesions, given the prevalence of pulmonic changes present in many cases of feline mycobacterial disease in GB it is not uncommon for bronchoalveolar lavage (BAL) to be performed (Albuquerque *et al.*, 2021). Cytological examination of BAL samples is key in attaining a diagnosis for some respiratory diseases *e.g.*, feline asthma (Trzil, 2020), which may be considered more likely as a cause for lesions identified on radiography or advanced imaging compared to mycobacteriosis. As pulmonic disease in cases of feline mycobacteriosis is typically interstitial, cytology on BAL samples may be unrewarding or reflect non-specific inflammation (Baral *et al.*, 2006), but ZN-positive BAL samples have been reported (Albuquerque *et al.*, 2021). Blood smear examination in cases of feline mycobacteriosis is rarely performed, but a case with circulating monocytes and neutrophils with intracellular negative-staining bacilli has been reported (Latimer *et al.*, 1997); bone marrow aspirates from this cat showed similar cytological changes.

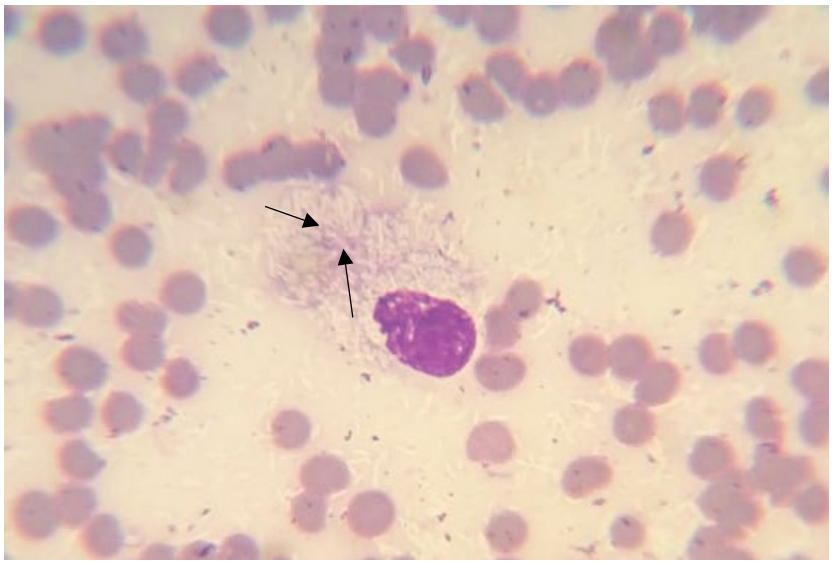

**Figure 1.7:** An FNA from the lesion shown in Figure 1.2, stained with Diff-Quik. Cytology was consistent with mycobacterial infection; a macrophage with abundant negative-staining 'ghost bacilli' (one such organism outlined with two black arrows) is shown against a background of erythrocytes. Image courtesy of Laura Vico MRCVS and Elise Cordell RVN.

### 1.1.4.3.1.2 Histopathology

Histopathology is an invaluable diagnostic technique in cases of feline mycobacterial disease (O'Halloran and Gunn-Moore, 2017), and an estimated 1% of all feline tissue biopsy samples submitted to diagnostic pathology laboratories shows changes suggestive of mycobacteriosis (Gunn-Moore *et al.*, 2013). Classically, feline mycobacterial lesions are typified by granulomatous to pyogranulomatous inflammation, with a dominance of epithelioid macrophages (Gunn-Moore *et al.*, 2011b) (Figure 1.8). A common feature of TB lesions in species such as cattle is the presence of Langhans MNGCs (Wangoo *et al.*, 2005), but these are rare in cases of feline TB (Kipar *et al.*, 2003, Gunn-Moore *et al.*, 2011b, Peterhans *et al.*, 2020). The reason for the lack of MNGCs in feline TB lesions is unknown, but it has been recently proposed that differences in species-specific host-pathogen interactions permit the formation of MNGCs under certain conditions (Queval *et al.*, 2021); these conditions may not be replicated in cases of feline TB. In contrast, MNGCs are more frequently reported in cases of NTM infection, but overall are still considered to be rare in cats (Appleyard and Clark, 2002, Malik *et al.*, 2002, Davies *et al.*, 2006, Meeks *et al.*, 2008, Lamagna *et al.*, 2009, Pekkarinen *et al.*, 2018). Another feature of mycobacterial lesions in cattle is mineralisation (Wangoo *et al.*, 2005); once again, feline mycobacterial lesions appear atypical as mineralisation is rarely reported (Gunn-Moore *et al.*, 2011b).

Grading systems to describe the histopathological features of TB lesions in cattle have been developed (Wangoo *et al.*, 2005), and successfully applied to other species such as wild boar (*Sus scrofa*) (García-Jiménez *et al.*, 2013) and fallow deer (*Dama dama*) (García-Jiménez *et al.*, 2012), but classification of feline TB lesions has not been attempted. However, there have been attempts to characterise the

histopathological appearance of lesions attributed to FLS. These were divided as showing a tuberculoid or lepromatous appearance (Malik *et al.*, 2002); tuberculoid lesions were those characterised by pyogranulomatous inflammation and a prominent lymphoid component, regions of necrosis, and few AFB, all suggestive of a strong cell-mediated immune response whereas lepromatous lesions are those with diffuse infiltration of foamy macrophages and abundant AFB, taken to indicate poor CMI. It has also been suggested that the histopathological appearance of lesions correlated with the underlying infecting organism, with tuberculoid lesions seen more frequent in young cats, attributed to *M. lepraemurium*, whereas lepromatous lesions were seen in older cats (Malik *et al.*, 2002), infected with *C.* 'M. tarwinense' or *C.* 'M. lepraefelis' (O'Brien *et al.*, 2017a, O'Brien *et al.*, 2017b). However, this classification has been debated by others as to its suitability (Kipar *et al.*, 2003, Davies *et al.*, 2006), and in a more recent study over half of *M. lepraemurium* infections were classified as lepromatous based on the presence of large numbers of AFB (O'Brien *et al.*, 2017c).

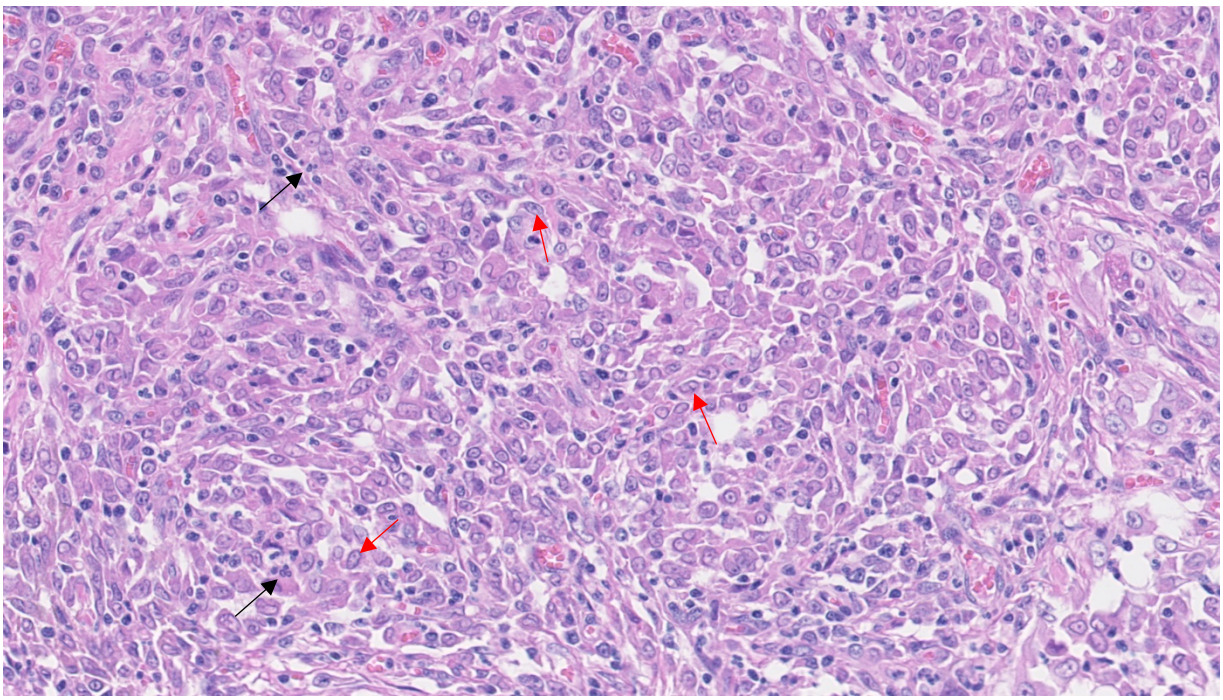

**Figure 1.8:** A haematoxylin and eosin (H&E)-stained section of a tissue biopsy from a case of interstitial granulomatous pneumonia due to *M. bovis* infection in a one-year-old FN Norwegian Forest Cat. The dominant inflammatory cell type present is the macrophage, many of which have an epithelioid phenotype (red arrows). There are smaller numbers of neutrophils (black arrows) interspersed throughout the granulomatous inflammation.

### 1.1.4.3.1.3 Special Stains

While identification of macrophages, in particular epithelioid macrophages, on cytological or histopathological examination, may be strongly suggestive of mycobacterial infection, or at least raise the possibility of this aetiological diagnosis to the reporting pathologist, visualisation of organisms morphologically consistent with mycobacteria is required to provide confirmation.

On cytology, negative-staining ghost bacilli may be identified as described previously (see *1.1.4.3.1.1 Cytology*). This is because mycobacteria do not retain Romanowsky stains due to the high lipid content of their cell wall (Chatterjee and Dey, 2014), hence appearing as a non-staining structure. Gram staining is considered unreliable for mycobacteria; they may be considered as weakly Gram positive, but results of Gram staining can be variable due to their complex, waxy cell wall not adequately retaining the crystal violet or safranin dyes (Fisher *et al.*, 1990).

Therefore, visualisation of mycobacteria within cytological and histopathological sections requires the use of acid-fast stains. The archetypal stain is ZN, although most protocols use a modification of this procedure such as FF. Acid-fast stains work by heat-fixing samples in the presence of carbol fuchsin, which allows for uptake of this dye by the cell wall of both acid-fast and non-acid-fast organisms. Washing the sample and then decolourising with an acid-alcohol solution results in loss of carbol fuchsin in non-acid-fast organisms, whereas mycobacteria will retain carbol fuchsin in their cell wall, as it is impervious to the acid-alcohol decolourisation due to the high lipid content. Counterstaining the sample *e.g.*, with methylene blue results in uptake of this dye by non-acid-fast cells, and the mycobacteria appear pink against this background (Carroll and Miller, 2016) (Figure 1.9). Modifications of the ZN method are required, as some species of mycobacteria have less mycolic acid in their cell wall, such as *M. leprae*, which can be lost when processed with organic solvents, resulting in a negative acid-fast stain. The FF method replaces organic solvents with vegetable oils, which retains the acid-fast property of mycobacteria. Another modification of ZN staining is the Kinyoun stain, or cold-ZN, which uses a higher concentration of carbol fuchsin and does not require heating of the sample (Carroll and Miller, 2016); similarly, this is designed to identify mycobacterial species that may not retain the carbol fuchsin stain due to their cell wall being “less acid-fast” (Fite *et al.*, 1947). Fluorescence microscopy can also be used to identify mycobacteria, namely staining with auramine O, or a mixture of auramine O and rhodamine B (auramine-rhodamine stain) (Van Hung *et al.*, 2007). Once again, these work on the principle of the dye successfully binding to the mycolic acid present within the mycobacterial cell wall, which will then fluoresce under excitation of light of an appropriate wavelength. Fluorescent staining is more sensitive than conventional special stains for the identification of mycobacteria (Kommareddi *et al.*, 1984, Somosközi *et al.*, 2001), allowing for more rapid reading of slides and improved detection of cases. However, the associated costs of fluorescent microscopes and other required equipment may place the use of these techniques out of reach for some veterinary diagnostic laboratories (Stewart and Giannini, 2016). Finally, immunohistochemistry (IHC) using anti-mycobacterial antibodies has been employed to identify mycobacteria within samples (Solomon *et al.*, 2017, Crothers *et al.*, 2021), though this is not routinely performed in veterinary diagnostic laboratories.

Despite the range of acid-fast stains available, the sensitivity of these methods for identifying mycobacteria within feline tissue samples is variable. In one study, only 30% of FFPE tissue samples with changes suggestive of mycobacteriosis were AFB-positive (Gunn-Moore *et al.*, 2013), whereas another study identified AFB in all 45 samples examined (Gunn-Moore *et al.*, 2011b), although AFB were rare in nearly half (47%) of the sections. Comparisons between staining methodologies for feline mycobacterial samples have not been published, although it has been suggested that modified ZN

stains, such as FF, are more sensitive for the detection of mycobacteria in samples from fish (Romano *et al.*, 2020) and humans (Crothers *et al.*, 2021) compared to traditional ZN staining. It has been proposed that ZN-negativity may arise from changes in the composition of the mycobacterial cell wall, particularly in states of latency in humans (Seiler *et al.*, 2003), as organisms were identified using IHC. Therefore, negativity on acid-fast staining does not rule out mycobacteriosis.

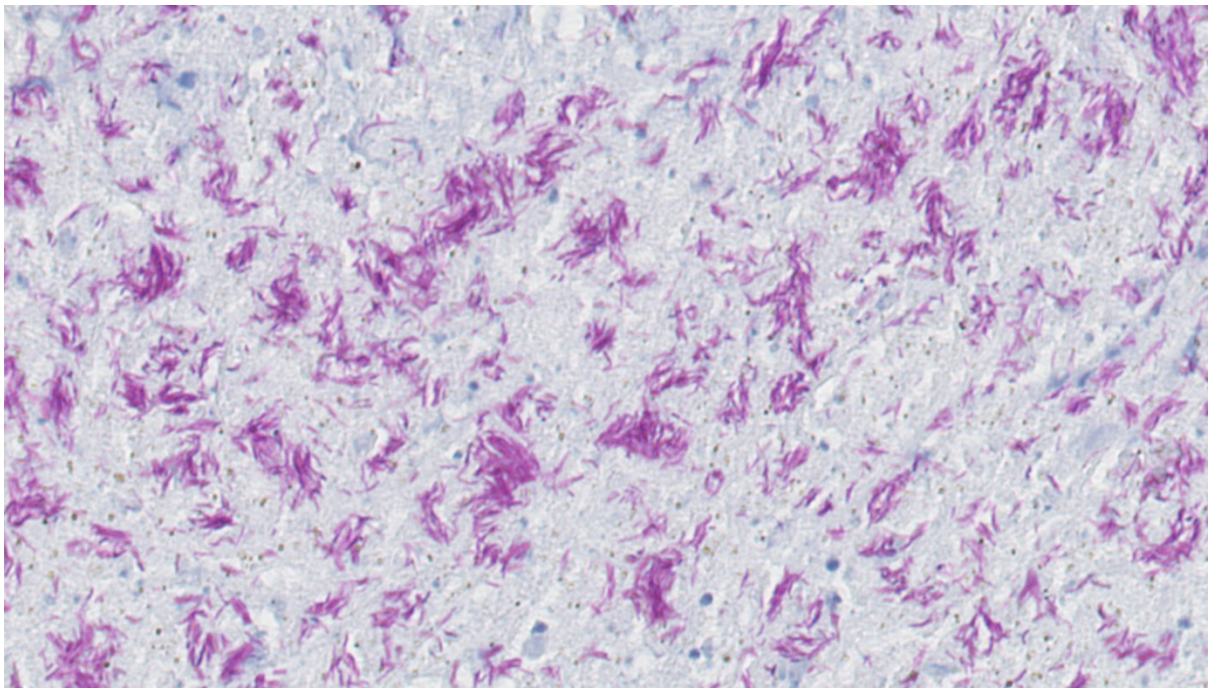

**Figure 1.9:** Abundant numbers of AFB within a region of necrosis in a choroidal lesion, identified using ZN staining. This cat was diagnosed with *M. bovis* infection on PCR of the FFPE tissue block (Case 8, Table 2.4).

It has been considered that the number of organisms or morphology of bacteria may facilitate discriminating between infections with different species of mycobacteria. For example, *M. microti* may appear as S-shaped on acid-fast staining (van Soolingen *et al.*, 1998), which could help differentiate this pathogen from other members of the MTBC (Brodin *et al.*, 2002). However, these differences may be subtle and easily missed, so morphology alone cannot be relied upon for an accurate diagnosis of *M. microti* (van de Weg *et al.*, 2019). One limitation of discriminating between mycobacterial species in feline infections based on the number of organisms is the lack of consistency with how this is quantified or scored; terms such as paucibacillary or multibacillary are frequently used but definitions differ between studies (O'Brien *et al.*, 2017b, Stavinohova *et al.*, 2019). In other studies, semi-quantitative methods have been used (Gunn-Moore *et al.*, 2011b); this investigation showed that cases of feline *M. bovis* infection presented with more AFB than cases of infection with *M. microti*. Therefore, a standardised approach to terminology and assessment would be beneficial in exploring the relationship between numbers of mycobacteria and the infecting species involved. Based on studies in cattle, TB has been considered a ‘paucibacillary’ infection where few AFB are identified (Wangoo *et al.*, 2005),

which has been adopted into our understanding of cases of feline TB. In contrast, feline NTM infections have been associated with abundant AFB (Malik *et al.*, 2013, O'Brien *et al.*, 2017a); consequently, histopathological reports for cases of feline mycobacteriosis with lots of ZN-positive organisms have often ruled out a diagnosis of TB in favour of NTM infection, particularly non-zoonotic organisms attributed to FLS based on this singular feature, which has been questioned (O'Halloran *et al.*, 2016).

Finally, there must be consideration to other acid-fast organisms such as *Nocardia* species (spp.) and *Rhodococcus* spp. Both nocardiosis and rhodococcosis have been reported in cats (Fairley and Fairley, 1999, Malik *et al.*, 2006, Aslam *et al.*, 2020), but these are rare compared to feline mycobacteriosis. Furthermore, the morphological appearance of organisms can help distinguish between these different bacterial pathogens; *Nocardia* spp. typically show branching morphology (Malik *et al.*, 2006), while *Rhodococcus* spp. are small coccobacilli (Fairley and Fairley, 1999). Both are also Gram positive, assisting the differentiation of these species from mycobacteria.

#### 1.1.4.3.2 Pathogen Detection

While cytology and/or histopathology in conjunction with special staining can provide an aetiological diagnosis of mycobacterial infection, it cannot provide a species-level identification of which mycobacterial organism is causing disease. Tests of pathogen detection provide one means by which this can be accomplished. Historically, specialist mycobacterial culture has been performed, although there are limitations to this approach and as such, there is an increasing scope of molecular diagnostic options available.

##### 1.1.4.3.2.1 Mycobacterial Culture

Culture of mycobacteria can be notoriously challenging for both human and veterinary infections alike. Non-sterile samples need to be decontaminated prior to setting up cultures, and samples may also require pre-treatment with antimicrobial agents to suppress the growth of other bacteria and fungi (Caulfield and Wengenack, 2016). It is advisable to culture mycobacteria in both solid and liquid media (Forbes *et al.*, 2018); there are many different types of media that can be used with differing chemical compositions that may be preferable for the recovery of different species of mycobacteria.

Mycobacterial culture at a reference centre such as the APHA Weybridge laboratory remains the reference standard diagnostic test for cases of mycobacteriosis in companion animals in GB (Middlemiss and Clark, 2018). However, one study reported than less than half (47%) of all feline samples submitted to APHA for specialist mycobacterial culture were positive (Gunn-Moore *et al.*, 2011a), indicating a major limitation of culture as a diagnostic test for feline mycobacteriosis. This is

likely to arise for several reasons. Firstly, culture systems used by the APHA are optimised for the growth of *M. bovis* (for the diagnosis of bTB) and these may not support the growth of more fastidious organisms seen in cases of feline mycobacteriosis (Gunn-Moore *et al.*, 2011a). Secondly, cultures for *M. bovis* are typically read at six weeks, whereas *M. microti* requires extended culture and may take up to three months for colonies to appear (Smith *et al.*, 2009). Thirdly, some species of mycobacteria require additional supplementation to support their growth such as mycobactin J for MAP and *M. genavense* (Coyle *et al.*, 1992, Stevenson, 2015). Finally, some species of mycobacteria that cause disease in cats are almost impossible to cultivate on media, such as *M. lepraemurium*; successful growth of this pathogen on culture media has been very rarely reported (Pattyn and Portaels, 1980, Ghielmetti *et al.*, 2021).

Culture also requires the submission of fresh tissue samples to the laboratory, which can be a limiting factor. As mycobacterial disease may not be suspected by the veterinary surgeon, a cat presenting with a cutaneous nodule or enlarged lymph node may have the lesion removed in its entirety and fixed in formalin for histopathological examination. This can provide a diagnosis of mycobacterial infection, but there is no longer fresh material available for culture. Additionally, if treatment is not initiated until the results of culture are available, the cat's clinical condition may have substantially deteriorated and achieving clinical resolution may no longer be possible. Therefore, alternative, more rapid diagnostic approaches are required.

#### 1.1.4.3.2.2 Nucleic Acid Tests

Molecular diagnostics *i.e.*, those that detect nucleic acids, can be used on cultured organisms to provide definitive species identification of isolates (Simner *et al.*, 2015), but they can also be applied directly to clinical samples, thus allowing a more rapid diagnosis of mycobacterial infection. These methods can be used to describe the molecular epidemiology of mycobacterial infections, which is important for the investigation of outbreaks of disease (O'Halloran *et al.*, 2020).

In the UK, commercially available molecular methods for the diagnosis of feline mycobacteriosis are those used for diagnosing mycobacterial infections in humans. This is a two-step process; firstly, following the extraction of nucleic acids from clinical samples PCR is performed to detect the presence of the 16S ribosomal ribonucleic acid (rRNA) gene found in all mycobacteria, and for the presence of MTBC-specific DNA. If MTBC DNA is not identified, the 16S rRNA product will be sequenced and compared to those deposited in online databases to assign a mycobacterial species (The Leeds Teaching Hospitals NHS Trust, 2021). Where MTBC DNA is identified, further testing is required to determine which species of the MTBC is causing disease; this is of much greater importance for cases of feline TB where disease can arise from infection with *M. microti* or *M. bovis* (Gunn-Moore *et al.*, 2011a), compared to human TB where approximately 99% of cases in high income countries are due to infection with *M. tuberculosis* (de La Rua-Domenech, 2006, World Health Organization, 2019b). This

is accomplished using a line probe assay (LPA), namely the GenoType MTBC test (Hain Lifescience GmbH, Nehren, Germany) (Richter *et al.*, 2003); other LPAs include the Inno-LiPA Mycobacterium (Innogenetics, Ghent, Belgium) (Mijs *et al.*, 2002) and the Speed-Oligo Mycobacteria (Vircell S. L., Granada, Spain) (Lara-Oya *et al.*, 2013). The methodology behind LPAs is that nitrocellulose strips are impregnated with specific probes, typically targeting the 16S and 23S rRNA DNA but other gene targets such as insertion sequence (IS)*6110* or the 16S-23S rRNA internal transcribed spacer (ITS) region may also be used. The extracted and amplified DNA from clinical samples is applied to the strip and if there is DNA complementary to the probe it will hybridise, producing a colorimetric band (Richter *et al.*, 2003, Caulfield and Wengenack, 2016). For the GenoType MTBC Test, the banding pattern obtained is unique for each species of the MTBC, allowing for species-level identification (Loiseau *et al.*, 2019). While the availability of this test improved the ability to diagnose mycobacterial infections in cats, there are some limitations. For example, the sensitivity of the test on FFPE tissues is poor compared to fresh tissues (Reppas *et al.*, 2013), but FFPE tissues may be the only sample available in cases of feline mycobacteriosis, undergoing two rounds of testing in cases of TB may be financially limiting for some owners, and these tests are not validated for use on feline clinical samples. Therefore, there is a requirement to have a veterinary diagnostic specific to the pathogens of interest for cases of feline mycobacteriosis, and that will have better sensitivity on the most readily available diagnostic material *i.e.*, FFPE tissues.

In-house molecular testing can also be performed, such as conventional PCR or real time/quantitative PCR (qPCR). Targets that are conserved across all species of mycobacteria, such as 16S rRNA, the 65kDa heat shock protein (*hsp65*) (Telenti *et al.*, 1993) and the 16S-23S rRNA ITS (Park *et al.*, 2000) may be used to confirm the presence of mycobacteria within a sample, and subsequent sequencing of these products may allow for identification of the species of mycobacteria. However, the sequences for these parts of the genome are identical across the MTBC, therefore further testing is required to obtain a species-level diagnosis. At 441 base pairs (bp) the *hsp65* product, also called the Telenti fragment, may be too long to reliably extract from FFPE tissues, reducing its usefulness for diagnosing feline mycobacterial infections.

Another approach is to perform a multiplex assay; this may involve a conserved target across all species of mycobacteria to confirm the presence of the organism in a clinical sample, and then complex-level testing to narrow down which type of mycobacteria is present. For the MTBC, IS*6110* (Costa *et al.*, 2014) or *mpb70* (Lorente-Leal *et al.*, 2019) may be used as gene targets, while IS*1311* can be used to identify all subspecies of *M. avium* (Shin *et al.*, 2010). To distinguish between members of the MTBC, parts of the genome that have been lost across species, termed a region of difference (RD), are attractive targets for the species-level identification of pathogens (Costa *et al.*, 2014). For example, RD9 can be used to determine whether a clinical sample is positive for *M. tuberculosis* or any other member of the MTBC, as only *M. tuberculosis* is RD9 positive. Similarly, *M. microti* and *M. bovis* BCG can be discriminated from other MTBC as they are both RD1 mutant deletions, although the exact part of RD1 that is deleted differs across these two organisms; these can be termed $RD1^{mic}$ and $RD1^{bcg}$, respectively (Brosch *et al.*, 2002, Pym *et al.*, 2002). As for the MTBC, the different *M. avium* subspecies can be

differentiated through targets that are specific to individual organisms (Shin *et al.*, 2010). For organisms beyond the MTBC and *M. avium*, a wide range of primers (and in the case of qPCR, probes) for different targets have been described (Elsner *et al.*, 2008, Fyfe *et al.*, 2008, O'Brien *et al.*, 2017b).

Another approach to diagnosing mycobacterial infections through pathogen detection is using fluorescence *in situ* hybridisation (FISH). This involves the binding of a fluorescently labelled nucleic acid probe to a specific complementary target DNA or RNA sequence in the sample of interest, which can then be visualised with fluorescence microscopy (Prudent and Raoult, 2019). This technique can be used on culture material, fresh tissues and FFPE biopsy samples (Yuan *et al.*, 2015, Alamri *et al.*, 2017), highlighting the diversity of FISH, but the application of this technique to FFPE tissues has only recently been adopted and there may still be technical challenges when processing samples which could compromise the performance of this technique as a diagnostic tool (Zordan, 2011, Yu *et al.*, 2019). The use of FISH for diagnosing mycobacteriosis has been employed in human medicine, with probes targeting the 16S or 23S rRNA to discriminate between MTBC and NTM infections (Stender *et al.*, 1999, Hongmanee *et al.*, 2001, Prudent and Raoult, 2019). However, current methods cannot distinguish between infection with members of the MTBC (Shah *et al.*, 2017), which somewhat precludes the use of FISH for the diagnosis of cases of feline mycobacteriosis given the requirement to differentiate between infection with *M. bovis* or *M. microti*. Similarly, not all species of NTM are identified by currently available NTM FISH probes, and there are a limited number of probes for the species level diagnosis of NTM infections (Lefmann *et al.*, 2006, Selvaraju *et al.*, 2008). Another consideration is that there is no amplification of DNA in FISH, so samples with low numbers of mycobacteria may not be detected. Despite these current limitations, FISH may prove to be a useful diagnostic tool for feline mycobacterial infections given further research.

Other molecular methods that have been used of identifying species within the MTBC include RFLP analysis, which exploits variations in highly similar sequences across mycobacterial species or complexes to assign a species-level diagnosis on digestion of the target sequence with an appropriate restriction enzyme (Van Embden *et al.*, 1993, Picardeau *et al.*, 1997, Van Soolingen *et al.*, 1998, van Soolingen *et al.*, 2001), and spoligotyping (spacer oligonucleotide typing), which uses a hybridisation technique to identify the presence of DNA spacer sequences in the direct repeat (DR) locus (Kamerbeek *et al.*, 1997). Recently, high resolution melting (HRM) assays have been described for the species-level identification of members of the MTBC (Landolt *et al.*, 2019a, Landolt *et al.*, 2019b), which exploit the fact that single nucleotide polymorphisms (SNPs) in genes such as *gyrB* will result in different melting temperatures of double stranded DNA bound to a fluorescently labelled dye; from this the mycobacterial species can be identified. These HRM assays have also been described previously for the differentiation of MTBC and NTM infections (Issa *et al.*, 2014); to date, HRM has not been trialled on feline mycobacterial samples. Another PCR-based molecular typing mechanism is mycobacterial interspersed repetitive unit-variable number of tandem repeats (MIRU-VNTR) assessment. The MIRU-VNTR method determines the number of MIRUs across 24 different loci (Supply *et al.*, 2006) and is reportedly less labour intensive compared to RFLP analysis (Jonsson *et al.*, 2014). Finally, whole genome sequencing (WGS) of mycobacteria can be performed, allowing for species and strain

identification, determination of drug susceptibility based on known genetic mutations associated with resistance, and the identification of outbreaks (Price-Carter *et al.*, 2018, Meehan *et al.*, 2019). Typically, WGS is performed on cultured isolates of mycobacteria, but WGS of mycobacterial species that cannot be readily grown in culture systems has been successfully accomplished (Benjak *et al.*, 2017). This can allow for the identification of species-specific DNA targets which could facilitate more cost-effective methods of diagnosis.

#### 1.1.4.3.2.3 Matrix-Assisted Laser Desorption Ionisation-Time of Flight Mass Spectrometry

An emerging diagnostic tool for infectious diseases is the use of matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry. This technique has been used for the identification of microorganisms from crude cell lysates of cultured samples but is now being used on clinical samples. Once again, mycobacterial specimens require special preparation in order to disrupt the cell wall to obtain a suitable sample for MALDI-TOF analysis (El Khéchine *et al.*, 2011), but this appears to be an attractive diagnostic tool for mycobacterial infections (Lefmann *et al.*, 2004, Ceyssens *et al.*, 2017). Briefly, the sample to be analysed is mixed or coated with an energy-absorbent organic matrix. As the matrix dries and crystallises, so does the sample. A laser beam is applied to the sample, resulting in desorption and ionisation of molecules to eventually produce singularly protonated ions, which are then accelerated to a TOF detector at a fixed potential. The ions separate based on their mass-to-charge ratio, and the time taken for the ions to travel the length of the flight tube is captured by the detector. This results in a peptide mass fingerprint (PMF) spectrum, which can be used to identify the microorganism by comparing the PMF of the unknown sample to spectra deposited in databases (Singhal *et al.*, 2015). Reports of MALDI-TOF analysis for diagnosing feline mycobacterial infections is rare, but it has been used to diagnose a case of *M. abscessus* infection (Munro *et al.*, 2021).

#### 1.1.4.3.3 Immunodiagnostic Assays

An alternative approach to diagnosing cases of mycobacterial infection is assessment of the host immune response to the pathogen, rather than directly identifying the bacteria itself. This relies on the detection of biomarkers in biological matrices, and these immunodiagnostic assays can be particularly useful when there are no readily accessible lesions that can be sampled for tests of pathogen detection, or the organism is difficult to isolate. Immunodiagnostic assays fall into tests of CMI or humoral immunity.

#### 1.1.4.3.3.1 Interferon-Gamma Release Assay

One test of CMI for the diagnosis of feline mycobacteriosis is the IGRA. It is an adaptation of the test initially developed for the diagnosis of TB in cattle that were not identified as positive using traditional testing methods, namely tuberculin skin testing (TST) (Plackett *et al.*, 1989, Wood *et al.*, 1990) (see *1.1.4.3.3.2 Tuberculin Skin Testing*), and it is now an approved test for the diagnosis of bTB in the UK (World Organisation for Animal Health (OIE), 2018). Compared to TST, the bovine IGRA is more sensitive (de La Rua-Domenech *et al.*, 2006) and allows for earlier detection of infection (Pollock and Neill, 2002, Pollock *et al.*, 2005), but it will still miss some cases of bTB and it has poorer specificity (de La Rua-Domenech *et al.*, 2006).

In cases of infection, antigen-presenting cells (APCs) will show mycobacterial antigens to T-lymphocytes, stimulating them to release a host of cytokines and chemokines, which includes IFNγ (O'Garra *et al.*, 2013), driving the immune response against the mycobacteria. The IGRA utilises the fact that an antigen-specific memory T-cell response is generated in cases of infection and repeat stimulation of circulating memory T-cells with mycobacterial antigens will result in further release of IFNγ into tissue supernatant (Pai *et al.*, 2004). An enzyme-linked immunosorbent assay (ELISA) is then performed on the supernatant to detect the presence of IFNγ, which is taken as a correlate for mycobacterial infection (Pathan *et al.*, 2001).

The feline IGRA was developed for testing cats where mycobacterial disease is strongly suspected (Rhodes *et al.*, 2008b). In this assay, peripheral blood mononuclear cells (PBMCs) are isolated from a heparinised blood sample rather than the test being performed on whole blood as is the case in cattle, as isolated PBMCs responded better than whole blood to simulation with mitogen which is used as the positive control (Rhodes *et al.*, 2008a). The PBMCs are incubated with a panel of antigens for four days, followed by IFNγ ELISA on the supernatants and the results are interpreted accordingly (Rhodes *et al.*, 2008a, Rhodes *et al.*, 2011). A positive and negative control are included, using a non-specific mitogen and cell culture media only, respectively. With respect to antigens both purified protein derivative (PPD) from *M. avium* (PPDA) and PPD from *M. bovis* (PPDB) are used as separate test conditions in the feline IGRA, in addition to an antigenic cocktail of early secreted antigenic target 6kDa (ESAT-6)/culture filtrate protein 10kDa (CFP-10, ESAT-6/CFP-10). The comparative PPD response is used to identify whether infection is attributable to an MTBC pathogen, characterised by a greater IFNγ response to PPDB than PPDA, or infection with NTM, where the PPDA response exceeds that to PPDB (Rhodes *et al.*, 2011). Inclusion of the ESAT-6/CFP-10 antigen cocktail allows for the differentiation of *M. bovis* from *M. microti*; this is because *M. microti* has lost the part of the genome that encodes these two proteins, RD1, whereas it is present in *M. bovis* (Pym *et al.*, 2002, Orgeur *et al.*, 2021).

Test performance data of the feline IGRA is limited, but it is currently suggested that the sensitivity for detecting MTBC infections is 70-100% depending on test interpretation (Rhodes *et al.*, 2011), whereas sensitivity for *M. avium* infections is only 50% (Rhodes *et al.*, 2008a). If there is no response to any of the mycobacterial antigens this has been interpreted as infection with a non-*M. avium* NTM (O'Halloran

and Gunn-Moore, 2017). Inclusion of the ESAT-6/CFP-10 antigen cocktail provides good discriminatory power to identify infection with either *M. bovis* or *M. microti* in cats that have a PPDB-biased response, but of note is that 20% of cats infected with *M. bovis* do not generate an ESAT-6/CFP-10 specific immune response (Rhodes *et al.*, 2011). This is comparable to results in cattle, where approximately 18% of cattle with *M. bovis* infection do not generate a specific IFNγ response to this antigenic cocktail (Buddle *et al.*, 2001, Vordermeier *et al.*, 2001, Vordermeier *et al.*, 2016). Also of note is that some species of NTM encode ESAT-6 and CFP-10 orthologues, namely *M. kansasii*, *M. szulgai*, *M. marinum*, and *M. riyadhense* (van Ingen *et al.*, 2009), and it has been shown that cattle infected with *M. kansasii* can generate an IFNγ response to stimulation with ESAT-6/CFP-10 (Waters *et al.*, 2006b). Feline infections with *M. kansasii* have been reported, but ESAT-6/CFP-10-specific immune responses were not identified (Černá *et al.*, 2020).

The feline IGRA has advantages over culture and PCR in that it has the most rapid turnaround time from sample submission to obtaining a result, and it can be used in cases of mycobacteriosis where there are no lesions that can be readily sampled for culture and/or PCR (Stavinohova *et al.*, 2019, Albuquerque *et al.*, 2021). It has also been utilised as a screening test for cats without signs of active mycobacterial disease but have either been exposed to cases of mycobacteriosis or a known risk factor for disease (O'Halloran *et al.*, 2020) (see *1.1.7 Asymptomatic Cats*).

#### 1.1.4.3.3.2 Tuberculin Skin Testing

The mainstay for the diagnosis of bTB in GB is TST, and it remains the international standard for *ante-mortem* diagnosis of bTB (de La Rua-Domenech *et al.*, 2006). In brief, intradermal injection with tuberculin – a crude protein extract from mycobacterial culture supernatant also referred to as PPD – elicits a delayed-type hypersensitivity (DTH) response if the animal has been previously sensitised to mycobacterial antigens. This reaction takes approximately 48-72 hours to develop and is mediated by sensitised T-cells (Thorns and Morris, 1983), and the swelling at the site of injection can then be measured. In the case of bTB, cattle may be injected with just bovine tuberculin/PPDB, or a comparative assay can be employed where both PPDB and PPDA are injected at separate sites (de La Rua-Domenech *et al.*, 2006). This comparative assay is more specific for diagnosing *M. bovis* infection because exposure to NTM can result in a cross-reactive response to PPDB (Hope *et al.*, 2005), so using both PPDA and PPDB increases the likelihood of identifying a DTH response specific to infection with a MTBC pathogen (Bezos *et al.*, 2014).

While TST is of use in cattle, it is not recommended for use in cats. One study showed that in a population of cats with culture-confirmed *M. bovis* infection there were no positive responses on TST (Snider *et al.*, 1971), while another study showed a lack of TST response in cats 60 days post-infection with *M. bovis* BCG (Legendre *et al.*, 1977). Use of TST as a screening tool for cats deemed at high risk of *M. bovis* infection failed to identify any positive results, although no lesions consistent with

mycobacteriosis were identified in any of these animals (Kaneene *et al.*, 2002). However, positive TST responses were reported in cats that had received two rounds of intradermal injection with a heat killed *M. bovis* preparation (*M. bovis* 'sensitinogen') approximately 99 and 123 days prior (Fenton *et al.*, 2010), but this experimental model may not reflect the processes in natural feline mycobacterial infections.

The role of TST for boosting antibody responses in cattle infected with *M. bovis* has been explored, and it has been shown that the detection of infected cattle using antibody-based assays increased following intradermal injection with PPDB (Casal *et al.*, 2014). In addition to this, IGRA responses are boosted in humans following TST (van Zyl-Smit *et al.*, 2009). Whether this is also true of cats has not been explored to date.

#### 1.1.4.3.3.3 Antibody-Based Diagnostics

The traditional paradigm of the immune response in mycobacteriosis, especially TB, is that CMI develops first and is important for the control of infection, but as the T helper type (Th) 1 response starts to wane there is progression of disease and generation of a Th2 response, which can be identified through the detection of mycobacterial-specific antibodies in biological matrices (Ritacco *et al.*, 1991, Pollock and Neill, 2002, Pollock *et al.*, 2005). This Th2 response would be associated with a switch towards humoral, antibody-based, immune responses suggested to occur late in the infection process. However, there is evidence that antibody responses can be detected as early as five days post-infection in cattle (O'Loan *et al.*, 1994), and that the humoral immune response may play a more important role in the immunopathogenesis of mycobacterial infection than has been previously thought (Achkar *et al.*, 2015). Despite this, the use of antibody-based diagnostics for identifying cases of TB in humans is not recommended (Steingart *et al.*, 2011), although they are still commonplace in some regions with a high incidence of disease such as India (Steingart *et al.*, 2012).

At present, there are no commercially available antibody-based tests for the diagnosis of feline mycobacteriosis. Where antibody testing has been performed, the results have been mixed. Rapid tests to detect antibodies against a combination of different antigens (ESAT-6, CFP-10, ESAT-6/CFP-10 fusion, MPB83, and Acr1) showed positive results in 67% and 90% of cats experimentally and naturally infected with *M. bovis*, respectively (Fenton *et al.*, 2010, Rhodes *et al.*, 2011). However, less than half of *M. microti*-infected cats gave antibody positive responses on rapid tests (Rhodes *et al.*, 2011). Importantly, no positive responses were identified in NTM-infected or negative control cats, suggesting good specificity of antibody-based tests for MTBC infections.

An alternative to rapid tests is the multi-antigen print immunoassay (MAPIA); this technique involves imprinting antigens onto a nitrocellulose strip, adding the sample of interest for testing, and then following this with immunochromatographic development to visualise a band in cases of a positive result (Lyashchenko *et al.*, 2000). While MAPIA responses for MPB83 antibodies were positive in 100% of

cats naturally infected with *M. bovis*, and 70% had antibodies against MPB70 (Rhodes *et al.*, 2011), antibodies to CFP-10 and ESAT-6/CFP-10 were only identified in 20% of cats, substantially lower than CMI responses to this antigenic cocktail (Rhodes *et al.*, 2011). In an experimental model of *M. bovis* infection, antibody responses on MAPIA testing were much less convincing (Fenton *et al.*, 2010). As for rapid tests, antibody responses to MAPIA antigens were reported in less than 50% of cats infected with *M. microti* and compared to rapid tests there was poorer specificity for MAPIA as positive antibody responses were identified in NTM-infected and negative control cats (Rhodes *et al.*, 2011).

An in-house ELISA to detect anti-PPDA and anti-PPDB antibodies was developed for screening a population of cats in-contact with an individual diagnosed with *M. bovis* infection; while a positive antibody response to PPDB was identified in 20% of these cats a similar response to PPDA was also reported (Kaneene *et al.*, 2002). Additionally, there were no signs of disease in any of these antibody-positive cats, suggesting these could have been false-positive results. Another study that utilised antibody tests to diagnose mycobacteriosis showed more promising results, with positive rapid test and MAPIA results in two clinically sick cats, one of whom had a culture-confirmed diagnosis of *M. bovis* infection, while the three healthy cats were negative (Ramdas *et al.*, 2015). Overall, the use of antibody testing for the diagnosis of feline mycobacteriosis is not recommended and is further limited by the lack of understanding of the feline immunological response to mycobacterial infections, and especially how the response differs between cases of infection with *M. microti* to *M. bovis*.

#### 1.1.4.3.4 Other Diagnostic Tests

While IFNγ has been the traditional biomarker for diagnosing cases of mycobacteriosis, there has been increasing research into whether measurements of alternative cytokines or chemokines could facilitate the diagnosis of mycobacterial disease (Yao *et al.*, 2017), for the discrimination between disease states (Tebruegge *et al.*, 2015), or to identify correlates of successful treatment (Clifford *et al.*, 2017). These studies have shown that in addition to IFNγ, the median concentration of IFNγ-inducible protein (IP)-10, tumour necrosis factor (TNF)-α, interleukin (IL)-1 receptor antagonist (ra), IL-2, IL-13 and macrophage inflammatory protein (MIP)-1β were increased in paediatric patients with TB compared to uninfected controls, and between disease states *i.e.*, discriminating between latent TB infection (LTBI) and active disease, the median concentration of TNFα, IL-1ra and IL-10 was higher in children with active disease (Tebruegge *et al.*, 2015). A similar study on adults with TB showed similar findings, with IP-10 and IL-2 responses higher in those with TB compared to healthy controls, in addition to granulocyte colony stimulating factor (G-CSF), granulocyte-monocyte colony stimulating factor (GM-CSF), IL-1α, B-cell attracting chemokine (BCA)-1 (also called CXC motif chemokine ligand [CXCL] 13), and eotaxin-1 (Yao *et al.*, 2017). Active disease could be differentiated from LTBI by measurement of antigen-unstimulated IP-10, MIP-1α, and soluble IL-2 receptor-α (sIL-2Rα), antigen stimulated vascular endothelial growth factor (VEGF), and monocyte-chemotactic protein (MCP)-3 (also called C-C motif ligand [CCL] 7), and both stimulated and unstimulated IL-8 (Yao *et al.*, 2017). Mycobacteria-specific cytokine responses

were also shown to decline over the course of treatment, in both active TB and LTBI (Clifford *et al.*, 2017); results varied greatly depending on antigen-stimulation conditions, although a decrease in IL-1ra was seen in treated cases of LTBI for all antigen-stimulation conditions. Some of these cytokines and chemokines have been investigated as to whether they can facilitate in the diagnosis of feline mycobacteriosis (O'Halloran *et al.*, 2018b); IL-13 was shown to be decreased in cats with mycobacteriosis compared to healthy controls, in contrast to what was reported in children (Tebruegge *et al.*, 2015). There was also a decrease in IL-4 and soluble Fas (sFas) in cats with mycobacterial disease, but neither of these were tested for in children. As for TB in humans, increases in GM-CSF, IL-2, and TNF-α in cats with mycobacteriosis were reported compared to healthy cats, as well as increased platelet-derived growth factor beta (PDGF-BB), IL-8, keratinocytes-derived chemokine (KC, also called cytokine-induced neutrophil chemoattractant [CINC] type-1), and regulated on activation, normal T-cell expressed and secreted (RANTES, also called CCL5). Interestingly, IFNγ was not increased in cats with mycobacterial disease compared to controls (O'Halloran *et al.*, 2018b). It was shown that measurement of certain cytokines and chemokines may help differentiate between infections with *M. bovis* and *M. microti*; PDGF-BB was increased in cats infected with *M. microti* while TNF-α concentrations were higher in *M. bovis*-infected cats. Cats with MTBC infections collectively had higher concentrations of GM-CSF, IL-2, and FMS-like tyrosine kinase 3 ligand (FTL3L) compared to those infected with NTM. Altogether, these findings may provide further assistance to differentiate between mycobacterial infections, but their application in a clinical setting is limited due to the equipment required to read plates not being routinely available in veterinary diagnostic laboratories.

One chemokine of interest that was not studied in cases of feline mycobacteriosis was IP-10 (also called CXCL10). This chemokine is secreted by APCs in response to stimulation by IFNγ (Liu *et al.*, 2011), driving proinflammatory responses. Detection of IP-10 in serum or in cytokine release assays has been performed in cases of mycobacterial disease in humans (Bamba *et al.*, 2019) and animals alike, including cattle (Waters *et al.*, 2012, Parsons *et al.*, 2016), warthogs (*Phacochoerus africanus*) (Roos *et al.*, 2018), and African buffalo (*Syncercus caffer*) (Bernitz *et al.*, 2019). Despite showing mixed results in cattle, parallel measurement of IP-10 alongside IFNγ facilitated the diagnosis of *M. bovis* infection in warthogs and African buffalo and may detect animals missed on IFNγ testing alone. This would be an attractive chemokine to investigate further in cases of feline mycobacteriosis.

Gene expression assays (GEAs) have also been investigated for the diagnosis of mycobacterial infections. These assays detect the amount of messenger RNA (mRNA) of a target gene relative to mRNA levels of a constitutively expressed gene to determine changes in gene expression in cases of infection. Such assays have been developed for wildlife species including badgers (*Meles meles*) and African buffalo (Sawyer *et al.*, 2007, Parsons *et al.*, 2012) (see *1.3.4 Diagnostic Testing)* and show promise for the diagnosis of *M. bovis* infection. Targets for GEAs are often IFNγ (Sawyer *et al.*, 2007) and genes induced by IFNγ such as CXCL9 (Sylvester *et al.*, 2017, Kerr *et al.*, 2020), but other targets such as TNF-α, IP-10 (also called CXCL10) and CXCL11 have also been investigated (Olivier *et al.*, 2017). The cross-species applicability of GEAs suggests they may be beneficial for diagnosing mycobacterial infections in cats.

### 1.1.5 Treatment and Monitoring the Response to Treatment

Treating cases of mycobacterial infection in cats is somewhat contentious (Human Animal Infections and Risk Surveillance (HAIRS) group, 2014), and in some countries, such as Italy, legislation does not permit the treatment of cats with *M. bovis* infection (Cerná *et al.*, 2019). The controversies surrounding the treatment of cases of feline mycobacteriosis, particularly TB, arise from concerns of sub-optimal treatment resulting in the generation of drug resistant strains of mycobacteria, the risk that cats may continue to be infectious to others, including humans, while on treatment, the risk of relapse post-treatment and the recurrence of clinical signs, and concerns about using antibiotics that some think should be reserved for use only in people. Despite these reservations, cases of feline mycobacteriosis can be treated and positive long-term outcomes have been reported (see *1.1.6 Prognosis*). Treatment requires prolonged courses of antimicrobial therapy, typically a combination of drugs with different mechanisms of action, and careful monitoring of the patient for side effects.

For cases of feline TB and most slow-growing NTM infections, treatment with a combination of rifampicin, a macrolide/azalide, and a fluoroquinolone is recommended *i.e.*, triple therapy (O'Halloran and Gunn-Moore, 2017) (Figure 1.10). The aim of treatment is to get the cat into clinical resolution or remission rather than curing the infection, partly because the dynamics of mycobacterial infections in cats and what truly constitutes ‘cure’ has not been defined. Treatment should be provided for a minimum of two months beyond the resolution of clinical signs (O'Halloran and Gunn-Moore, 2017). For cases presenting with only cutaneous or lymph node lesions, and no evidence of disseminated disease on imaging, treatment is typically given for a minimum of four months. If there is evidence of disseminated disease on imaging, such as pulmonic disease, or enlarged abdominal lymph nodes, treatment should be provided for at least six months (O'Halloran and Gunn-Moore, 2017). If lesions are present in sites with poorer antimicrobial penetration, such as bone, eyes, or the CNS, a longer course of treatment is likely to be required, and antimicrobials may need to be given at a higher dose than what is standard (Černá *et al.*, 2020). Treatment should be initiated as early as possible; initial therapy with at least pradofloxacin (a fluoroquinolone; given at a dose of 5mg/kg *per os* [PO] once every 24 hours [q24h]), ideally in combination with azithromycin (an azalide, given at 10-15mg/kg PO q24h), then rifampicin (10mg/kg PO q24h) is introduced once the species of mycobacteria is identified. If there are financial limitations for diagnostic investigations, it is advised to treat with this triple therapy protocol and make subsequent changes as needed based on the response to treatment.


**Figure 1.10:** A combined rifampicin and azithromycin capsule formulated for the treatment of cases of feline mycobacteriosis provides two of the core drugs that comprise the 'triple therapy' protocol. Other drug suppliers and compounding pharmacies can also manufacture and provide similar or related products.

Rifampicin is a key component in treatment protocols for cases of TB in humans (Grobbelaar *et al.*, 2019) as well as cats. It acts by binding to the β-subunit of DNA-dependent RNA-polymerase (RNAP); this binding inhibits the subunit of RNAP, preventing the elongation of RNA during transcription (Campbell *et al.*, 2001). Rifampicin is effective against replicating susceptible bacteria, but its activity against non-replicating organisms is greatly reduced or may be completely eradicated (Cho *et al.*, 2007, Jakkala and Ajitkumar, 2019); this is thought to be attributable to changes in the mycobacterial cell wall under hypoxic conditions restricting entry of rifampicin into the bacteria (Jakkala and Ajitkumar, 2019). Despite this, rifampicin has been shown to be suitable for the treatment of LTBI in humans (Ronald *et al.*, 2020), which may infer low-level replication of mycobacteria in cases of LTBI. There are some concerns with the use of rifampicin in cats, particularly due to the side effects of this drug. Rifampicin-related hepatotoxicity is a major concern; there can be a subclinical dose-dependent interference with bilirubin uptake, but there may also be an idiosyncratic hypersensitivity reaction resulting in hepatocellular injury, presenting as anorexia, nausea, and vomiting (Saukkonen *et al.*, 2006). Baseline haematology and serum biochemistry prior to starting treatment is recommended (see *1.1.4.1.1 Haematology and Serum Biochemistry*), followed by repeat bloods two weeks after starting rifampicin, and subsequent repeat testing if indicated due to the presence of signs of hepatotoxicity (O'Halloran and Gunn-Moore, 2017). To help mitigate the risk of some of these side effects, hepatoprotectants such as S-adenosyl-L-methionine (SAMe) or N-acetyl cysteine (NAC) may be used (Černá *et al.*, 2020); NAC has also been shown to have direct antimycobacterial activity *in vitro* and may therefore be of additional benefit for the treatment of mycobacterial infections (Amaral *et al.*, 2016). Cutaneous side effects are also reported with rifampicin use and in cats this can manifest as pruritus, erythema, and skin oedema (O'Halloran and Gunn-Moore, 2017). These can be managed or reduced using agents such as chlorphenamine, or topical hydrocortisone aceponate spray. Other side effects include hyperaesthesia, and the red-orange discolouration of bodily fluids which may be mistaken for haemorrhage (O'Halloran and Gunn-Moore, 2017). In addition to the side effects of this drug, rifampicin is a potent inducer of

cytochrome P450 3A4 (CYP3A4), which can influence the metabolism of other drugs used in the treatment of mycobacterial disease, resulting in sub-optimal concentrations (Shimomura *et al.*, 2015). Finally, rifampicin should always be used in combination with other drugs because resistance to this agent can be rapidly induced when used as monotherapy (Goldstein, 2014), contributing to the problem of drug resistant strains of mycobacteria.

The macrolide/azalide of choice for feline mycobacterial infections is azithromycin, as this can be given once daily, facilitating treatment over a long period of time (O'Halloran and Gunn-Moore, 2017). Azithromycin has been shown to achieve good distribution throughout body tissues (Girard *et al.*, 1987), and can achieve particularly high concentrations in alveolar macrophages (Stamler *et al.*, 1994). Macrolides/azalides inhibit protein synthesis by binding to the 50S ribosomal subunit (Kanoh and Rubin, 2010). However, macrolides/azalides are not used for the treatment of TB in humans, as it has been shown that *M. tuberculosis* is inherently resistant to azithromycin *in vitro* (Watt *et al.*, 1996), due to the presence of the *erm37* gene. Expression of this gene results in methylation of an adenosine nucleotide in the 23S rRNA, preventing the macrolide/azalide from binding to the ribosome, which drives resistance to some macrolides/azalides (Buriánková *et al.*, 2004). Further *in vitro* testing has shown high levels of resistance to azithromycin among *M. bovis* and *M. microti* isolates, but low levels of resistance to clarithromycin (Buriánková *et al.*, 2004). Further studies have suggested that additional mechanisms are present that drive macrolide/azalide resistance, and that while *M. tuberculosis* resistance to clarithromycin can be induced, *M. microti* appears to remain susceptible to this agent even with prior pre-incubation with sub-optimal concentrations of this drug (Andini and Nash, 2006). In contrast, macrolides/azalides have been shown to be of particular benefit for the treatment of *M. avium* infections in humans and are recommended in first line treatment protocols (Dautzenberg *et al.*, 1995, Griffith *et al.*, 2007). Clarithromycin has also been recommended as a drug of choice for the treatment of feline *M. avium* infections (Baral *et al.*, 2006). While comparative studies between clarithromycin and azithromycin for feline *M. avium* infections have not been undertaken, there is not thought to be any difference between agents for the treatment of human infections (Dunne *et al.*, 2000). Clarithromycin has also been used for the treatment of other feline NTM infections with success (O'Brien *et al.*, 2017b, O'Brien *et al.*, 2017c); however, there is evidence from humans to suggest that the combination of rifampicin and clarithromycin results in increased metabolism of the macrolide due to the induction of CYP3A4 to the point that serum levels of clarithromycin are inadequate for the treatment of mycobacterial infections (Shimomura *et al.*, 2015), which may result in treatment failure. Of note, clarithromycin was only given once daily in this study, whereas in cats it is given twice daily as standard and not just in cases of feline mycobacteriosis (O'Halloran and Gunn-Moore, 2017). Twice daily dosing with clarithromycin may increase levels above the minimum inhibitory concentration when used in combination with rifampicin for humans (Alffenaar *et al.*, 2010) Given it is currently given twice daily in cats this may result in suitable levels of clarithromycin for activity against mycobacteria in conjunction with rifampicin, though pharmacokinetic studies would be required to confirm this.

Pradofloxacin is recommended as the fluoroquinolone of choice for treatment of feline mycobacterial infections and it has been shown to be effective against rapidly-growing mycobacteria (RGM) (Govendir

*et al.*, 2011b). It has greater *in vitro* activity mycobacteria than other fluoroquinolones (Govendir *et al.*, 2011a), although results with moxifloxacin were comparable; moxifloxacin is almost identical to pradofloxacin and can be thought of as its human medicine counterpart (Blondeau, 1999, Silley *et al.*, 2007). Fluoroquinolones bind to topoisomerase II and IV, which both cleave DNA during DNA replication, disrupting DNA and protein synthesis and eventually results in bacterial killing in a concentration-dependent manner (Silley *et al.*, 2012, Sykes and Blondeau, 2014). Side effects of pradofloxacin include reversible myelosuppression and gastrointestinal signs such as vomiting and diarrhoea (Sykes and Blondeau, 2014). While enrofloxacin has been used for the treatment of mycobacterial infections in cats (Malik *et al.*, 2002, O'Brien *et al.*, 2017c), it can cause retinal degeneration and is not recommended for use in this species (Gelatt *et al.*, 2001).

Other drugs that may be used in the treatment of feline mycobacterial infections include doxycycline and clofazimine (Gunn-Moore, 2014). Doxycycline has been used to great effect for the treatment of infections with *M. smegmatis* and it may be suitable as monotherapy without the requirement for rifampicin (Malik *et al.*, 1994). Doxycycline may have some place in the treatment of *M. lepraemurium* infections (O'Brien *et al.*, 2017c), but it appears to be less effective for treating *M. avium* infections (Baral *et al.*, 2006). Clofazimine also appears to be effective for *M. avium* infections (Malik *et al.*, 2002, Baral *et al.*, 2006) and shows *in vitro* activity against RGM (Bennie *et al.*, 2015), although it may not be readily available for treating feline infections.

Ethambutol and isoniazid, both of which are integral components of the treatment of TB in humans, are rarely used in cases of feline mycobacteriosis due to the severe toxicities associated with these drugs (Gunn-Moore, 2014, O'Halloran and Gunn-Moore, 2017). They may be considered in cases that are non-responsive to first-line treatment, but cats must be carefully monitored carefully for side effects. Supplementation with pyridoxine could help limit or even prevent the neurological side effects associated with use of isoniazid (Glatstein *et al.*, 2018), but this has not been thoroughly evaluated in cats (Kanegi *et al.*, 2019).

While medical therapy is the mainstay of treatment for cases of feline mycobacteriosis, surgery may also be undertaken; however, this is not without risk. Where surgery has been performed without adequate prior control of infection, surgical wounds may dehisce (O'Halloran *et al.*, 2019, O'Halloran *et al.*, 2020) and revision surgery may not be possible. Extensive surgery has been successfully implemented in the treatment of panniculitis due to RGM following a course of antimicrobial treatment (Malik *et al.*, 1994), but this should be performed by an experienced reconstructive surgeon given the large amount of tissue that may need to be removed necessitating extensive surgical restoration. In some cases, surgery may be performed as part of the diagnostic investigation *e.g.,* enucleation in cats with ocular lesions (Stavinohova *et al.*, 2019), or complete surgical excision of singular cutaneous masses. Where a focal lesion has been removed with wide margins, this may prove to be curative, especially if infection is attributed to a species that does not cause systemic disease such as *M. lepraemurium* (O'Brien *et al.*, 2017c) (Figure 1.11). If MTBC organisms or *M. avium* are identified as causing singular cutaneous lesions surgical excision may be sufficient, but adjunctive antimicrobial

therapy to treat any remaining microscopic infection is advisable. Amputation is recommended in cases of articular TB (Lalor *et al.*, 2017), or where there is extensive osteomyelitis, given the difficulties of antimicrobial agents to penetrate bone and joints. Despite this, some cases of mycobacteriosis affecting the skeletal system have been successfully treated with medical therapy alone (Černá *et al.*, 2020).

Monitoring the response to treatment in cases of feline mycobacteriosis can be accomplished through repeat laboratory testing and imaging, where appropriate. If hypercalcaemia was reported at the time of diagnosis, further blood samples should be taken to ensure that the concentration of calcium returns to reference interval levels; this should resolve with appropriate antimicrobial therapy to treat the mycobacterial infection without the requirement for specific calcium-chelating agents. If lesions were identified on imaging, it is recommended to repeat this after at least four months of treatment to determine whether lesions have resolved at this point, and thus the final 'two-month lesion free' period of treatment can start (giving a total duration of six months treatment). If there are still lesions present on imaging, antimicrobial therapy should be continued, and imaging repeated on a two-to-three-month basis. Given the improved resolution and sensitivity of CT compared to standard radiography, lesions may still be identifiable at the point where resolution would have been achieved on radiography; this may reflect areas of fibrosis or lesions that are no longer active (Major *et al.*, 2018). Therefore, treating to the point of resolution on CT is not recommended. The use of diagnostics such as the IGRA or antibody tests for defining the response to treatment in cats has been poorly described (Rhodes *et al.*, 2011, Ramdas *et al.*, 2015, Major *et al.*, 2018), so it cannot be ascertained whether reversion to IGRA negativity should be the goal when treating such infections, or what the significance is of remaining IGRA positive despite the resolution of lesions.

Despite evidence to show that cases of feline mycobacteriosis can be successfully treated, euthanasia may be elected in some cases. This could be due to the potential zoonotic risk of infection (see *1.2 Public Health Risks*), the costs of treatment, concerns around providing long-term medication, and previous treatment failure or lack of response to therapy. Where euthanasia is performed, or if the cat dies naturally, they should be sent for cremation rather than burial to not contaminate the environment with mycobacteria.

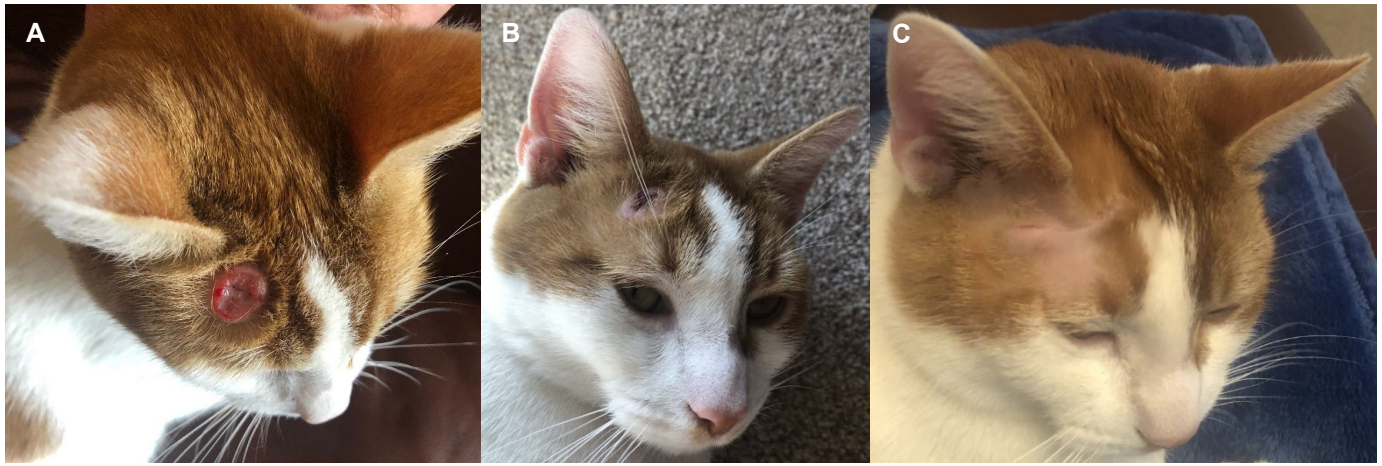


**Figure 1.11:** Successful treatment of a case of *M. lepraemurium* infection in a two-year-old MN DSH cat. At the time of presentation to the primary veterinary surgeon (PVS) there was a well demarcated, raised, alopecic, ulcerative cutaneous lesion rostral to the right ear base (A). An initial diagnosis of NTM infection was made on IGRA, and this was interpreted as being due to *M. avium*; treatment with rifampicin, azithromycin, and pradofloxacin was started. An FNA of the mass was taken and *M. lepraemurium* was diagnosed on PCR; it was decided to continue with rifampicin, azithromycin, and pradofloxacin 'triple therapy'. Approximately four months after starting treatment the lesion had reduced in size and was starting to crust over. Treatment was continued for one further month and the mass was surgically removed (B). Two weeks post-surgery and the surgical site has healed over with no signs of post-operative infection (C). Treatment was continued to give a total of six months of triple therapy. One month after this image was taken the owner reported the cat was doing well with no signs of recurrence of the mass. Images courtesy of the owner (Ms Haddon) and Bill Bowler MRCVS.

### 1.1.6 Prognosis

Outcomes in cases of feline mycobacteriosis are likely influenced by the underlying infecting species and possibly strain of mycobacteria, the extent of disease, implementation of an appropriate treatment protocol, and the host immune response to infection. Therefore, the prognosis for an individual case will depend on the combination of these factors. Another consideration has to be given to the terminology surrounding feline mycobacterial infections; there is no standardised approach to defining case outcomes in cats, unlike for cases of pulmonary TB (World Health Organization, 2013) or the recently proposed definitions for cases of ocular TB (OTB) (Agrawal *et al.*, 2019) in humans, which limits the ability to compare feline outcomes across different studies.

One study reported 42% of cats achieving complete remission of disease, however, only 16% of all cats in the study were treated with a triple therapy protocol, and 61% of all cats were treated for less than one month (Gunn-Moore *et al.*, 2011b). Of those that were treated with triple therapy 45% went into remission, and while this proportion was not substantially greater than the proportion of cats who achieved remission on other protocols (41%) it may have been the case that these cats who received triple therapy presented with more advanced disease, hence they were treated with a more aggressive protocol. A recurrence of clinical signs was reported in 63% of cats post-treatment, with progression to respiratory or systemic disease reported in 32% and 52% of cases, respectively (Gunn-Moore *et al.*, 2011b). Since triple therapy protocols have become the mainstay of treatment for cases of feline mycobacteriosis in GB, there are reports of 70-90% of cats achieving remission or long-term success for treatment of MTBC infections (O'Halloran and Gunn-Moore, 2017, Major *et al.*, 2018, Stavinohova *et al.*, 2019); however, both ‘remission’ and ‘long-term success’ have not been defined, which may prove limiting when relaying information regarding prognosis to clinicians and owners.

Despite this more encouraging data with regards to prognosis in cases of feline mycobacteriosis, the recent outbreak of raw food associated *M. bovis* infection in cats saw a fatality rate of 83% in the first identified cases (O'Halloran *et al.*, 2019). These cats presented with abdominal lesions as part of a severe, disseminated disease process which may have contributed to their worse outcomes. Additionally, where culture and genotyping was performed, it was found that these cats were infected with *M. bovis* 10:a, the genotype also associated with an outbreak of TB in a pack of working Foxhounds that also had a high case fatality rate (O'Halloran *et al.*, 2018a). This strain of *M. bovis* may be inherently more pathogenic than other *M. bovis* genotypes; further research is required regarding *M. bovis* genotypes and virulence in companion animal species, however, data from cattle would suggest differences in virulence between genotypes of *M. bovis* exist (Wright *et al.*, 2013).

Regarding NTM, there are reports of successful treatment of cases of *M. avium* infection in cats (Malik *et al.*, 1998, Baral *et al.*, 2006, Sieber-Ruckstuhl *et al.*, 2007, Kanegi *et al.*, 2019), but there are many smaller studies and individual case reports suggesting treatment of this infection is incredibly difficult, with many cases failing to respond to therapy or relapsing, eventually resulting in euthanasia

(Stevenson *et al.*, 1998, Barry *et al.*, 2002, Rivière *et al.*, 2011, Madarame *et al.*, 2017, Paharsingh *et al.*, 2020, Munro *et al.*, 2021). Another NTM species that appears difficult to treat is *C.* 'M. lepraefelis', with only 35% of cats achieving resolution; the prognosis was worse in those where oedema was noted (O'Brien *et al.*, 2017a). In contrast, cases of *M. lepraemurium* infection have high rates of resolution (89%), and a small number of cases appeared to resolve spontaneously without requiring antimicrobial therapy (O'Brien *et al.*, 2017c). This variation in outcomes demonstrates why it is important to obtain a species-level diagnosis of infection.

### 1.1.7 Asymptomatic Cats

Mycobacterial infections in cats are thought of as causing clinical disease, as this is the point at which it is identified by the owner and/or veterinary staff. However, the spectrum of mycobacterial infections is much more complex than simply infected and displaying clinical signs of disease, or uninfected. In humans, five categorical states of TB have been proposed (Drain *et al.*, 2018). These are:

- Eliminated TB infection: infection has been cleared by the host immune response, or microbiological cure has been achieved. The individual no longer harbours mycobacterial organisms but may continue to have immunological evidence of infection.
- LTBI: this has been defined by the WHO as "evidence of TB infection and no clinical, radiological or microbiological evidence of active TB disease" (World Health Organization, 2019b), but an alternative definition has been suggested of infection with viable mycobacteria, but progression to active disease is unlikely in the absence of significant immunological compromise.
- Incipient TB infection: infection with viable mycobacteria that is likely to progress to clinically active disease without intervention, but clinical signs, radiographic abnormalities and microbiological testing do not show signs of disease.
- Subclinical TB disease: infection with viable mycobacteria that is not causing compatible clinical signs, but for which there may be radiographic or microbiologic evidence of disease.
- Active TB disease: disease with clinical symptoms, radiographic abnormalities, and microbiological evidence consistent with TB.

While these definitions have been applied to cases of TB in humans, adapting and applying these categories to describe cases of feline mycobacteriosis is less straightforward. While active mycobacterial disease has been well described in cats, states such as latency, incipient infection, and subclinical disease have not been applied, in part because testing for immunological responses to mycobacterial antigens is unlikely to be performed in overtly healthy cats, or those not showing clinical signs suggestive of mycobacteriosis.

The diagnosis and definition of LTBI in humans can be thought of as a circular argument; LTBI is defined as an immunological response to mycobacterial infection in the absence of clinical signs or isolation of mycobacteria, and a diagnosis of LTBI is made by detecting an immunological response to mycobacterial antigens without evidence of active disease (Kiazyk and Ball, 2017). In humans, this can be achieved through TST, IGRA, or a combination of both tests (Torres-Gonzalez *et al.*, 2013). In cats, diagnosing LTBI (or latent mycobacteriosis, if considering both MTBC and NTM infections) could theoretically be achieved on IGRA testing.

The IGRA (see *1.1.4.3.3.1 Interferon-Gamma Release Assay*) was adapted for use in cats with signs of active mycobacteriosis, and current test interpretation is based on this underlying assumption that the index of suspicion for mycobacterial infection in the tested individual is high (Rhodes *et al.*, 2008b). However, it has since been used for screening populations of cats without signs of active disease, but who have either cohabited with an individual diagnosed with mycobacteriosis (by either IGRA, PCR or culture) or who have been exposed to a risk factor predisposing them to infection (O'Halloran *et al.*, 2019, O'Halloran *et al.*, 2020). This is because it is the only test that can be used on an asymptomatic cat, as both culture and PCR require sampling of a lesion from which mycobacteria can be isolated or detected. The consequence of this is: test results are obtained for cats where the index of suspicion for mycobacterial disease may be low, but the outcome may result in the veterinarian and owner having to make decisions such as whether to treat or euthanise an overtly healthy cat, despite the significance of a positive or negative result in this population of animals not being truly understood.

It has been suggested that if the IGRA is used as a screening test of asymptomatic cats, any positive result should be investigated further with thoracic and abdominal imaging to identify signs of structural disease (O'Halloran *et al.*, 2019). If a positive IGRA result is seen alongside signs of disease on imaging, treatment can be initiated, however, this raises questions as to what protocol should be used, what should be considered the endpoint of treatment, and what is the potential for generating drug-resistant strains of mycobacteria. If the IGRA is positive but there is no sign of disease on imaging, this further complicates matters. It remains unclear whether there will be progression to active disease, and whether the cat should be treated prophylactically. If antimycobacterial prophylaxis is considered, it needs to be decided what agents are the most appropriate for this. Alternatively, the decision to carefully monitor for subtle signs such as changes in body condition, body weight, and the respiratory rate and/or effort could be considered, and further investigation and treatment initiated at the point of observable disease. Another consideration is that a positive IGRA without signs of disease on imaging may indicate eliminated infection, which is likely to be difficult to conclusively prove in cats. Conversely, a negative IGRA may be interpreted as the cat not being infected, but it is not known how long the CMI response takes to develop in cats infected with mycobacteria, so if the cat was to be re-tested it may become IGRA positive. It has been suggested that a positive IGRA result will not be detected in a human with *M. tuberculosis* until eight weeks post-infection (Muñoz *et al.*, 2015), so this time scale could be considered for re-testing IGRA negative cats. Questions may also be asked as to whether the cat could be prophylactically treated with antimicrobials, which may be highly controversial given there is no clear

indication of the need to treat. These questions demonstrate that this is an important area of research in feline mycobacteriosis that has not been adequately addressed to date.

## 1.2 Public Health Risks

Mycobacteria can cause disease in both humans and animals; therefore, an understanding of feline mycobacterial infections is of importance not only for animal health, but also for human health. Globally, in 2018 around 1.4% of cases of TB in humans were due to infection with *M. bovis*, and around 1% of TB deaths were in cases of *M. bovis* infection (World Health Organization, 2019b). However there are concerns that cases of 'zoonotic TB' *i.e*., human infection with *M. bovis*, are under-reported (Olea-Popelka *et al.*, 2017), and there are marked differences in the distribution of cases of human *M. bovis* infection globally. The proportion of cases of human TB attributable to *M. bovis* infection in the UK is estimated at 0.5-1.5% (de La Rua-Domenech, 2006), whereas this figure can approach almost 30% in some low- and middle-income countries (Luciano and Roess, 2020). The impact of zoonotic TB has been recognised as an important threat which needs to be addressed as part of global impacts to end the TB epidemic (Dean *et al.*, 2018). For example, cases of zoonotic TB are more likely to present as extrapulmonary disease, rather than pulmonic disease, compared to infection with *M. tuberculosis* (Dürr *et al.*, 2013); as zoonotic TB may present with unexpected symptoms, cases may go unrecognised and not receive adequate treatment. Linked in with this is that *M. bovis* is naturally resistant to pyrazinamide (Sreevatsan *et al.*, 1997), which is one of the cornerstone drugs for treatment of *M. tuberculosis* infection in humans, so treating a case of zoonotic TB with inappropriate therapy may result in worse clinical outcomes (Dean *et al.*, 2018).

Overall, public health bodies consider the risk cats pose to humans in terms of zoonotic transmission of mycobacteria to be very low (Human Animal Infections and Risk Surveillance (HAIRS) group, 2014). However, it is recognised that cats may act as 'amplifier' hosts for mycobacteria, in particular *M. bovis*, and therefore should be considered a risk in terms of infecting other animals as well as humans (de La Rua-Domenech, 2006). Treating cases of *M. bovis* infection in cats is contentious for some (see *1.1.5 Treatment and Monitoring the Response to Treatment*) (Human Animal Infections and Risk Surveillance (HAIRS) group, 2014), so the zoonotic risk associated with any case of feline mycobacteriosis has to be considered by the veterinary staff and discussed with the owners, and where appropriate with public health authorities.

While legislation surrounding mycobacterial infections in companion animals in the UK is devolved to national governments, it is broadly identical across countries (*The Tuberculosis Control Order (Northern Ireland) 1999, The Tuberculosis (Scotland) Order 2005, The Tuberculosis (Wales) Order 2011, The Tuberculosis (England) Order 2014*). If *M. bovis* infection is suspected in a deceased cat, or *M. bovis* has been isolated in a laboratory from a submitted tissue sample, the relevant authority should be notified. Further investigations will be carried out as deemed necessary.

## 1.2.1 Human Risk Factors

Risk factors have been identified by the World Health Organization as increasing the risk of TB in humans. Most new cases of TB are attributed to five risk factors: HIV infection, diabetes mellitus, alcohol misuse, malnutrition, and smoking (World Health Organization, 2021). The risk of mycobacterial infection is also thought to be increased in people who have cystic fibrosis, kidney disease, are receiving immunomodulatory medications, are organ transplant recipients, are pregnant, or who are at extremes of age (Biet *et al.*, 2005, O'Halloran and Gunn-Moore, 2017). An occupational risk has been proposed for cases of zoonotic transmission, with farmers, abattoir workers and veterinary staff identified as being at increased risk of *M. bovis* infection, particularly through inhalation of infective aerosol droplets (Biet *et al.*, 2005, Posthaus *et al.*, 2011).

## 1.2.2 Cases of Zoonotic Transmission from Cats

Cases of cat-to-human transmission of mycobacteria are rare and often lacking genotypic confirmation (Lewis-Jonsson, 1946), but two cases of microbiologically and genetically confirmed *M. bovis* infection in humans originating from a cat have been recently reported in GB (O'Connor *et al.,* 2019), providing conclusive evidence that feline mycobacterial infections do pose a risk to human health. One of these individuals who developed active TB tested positive for LTBI six months prior to presentation but declined treatment, while the second person with active TB due to *M. bovis* infection declined initial screening. These two individuals were from separate households. Both individuals had been in close contact with a cat with a culture-confirmed *M. bovis* infection that had a purulent draining wound; the wounds were cleaned without appropriate hygiene measures (Gunn-Moore and Lalor, 2015). Therefore, purulent lesions should be considered a risk factor for the transmission of *M. bovis* from cats to humans and such lesions should be handled with care including the wearing of surgical gloves and masks.

An historical case of an individual testing positive on TST following contact with *M. bovis*-infected cats has been reported, but this person never developed active TB (Isaac *et al.*, 1983). Additionally, this person had previously received a BCG vaccination, which could result in a false-positive skin test result (Cohn, 2001). Positive results on tests of CMI were reported in a small number of owners and vets of cats implicated in an outbreak of *M. bovis* infection following consumption of a commercial raw food diet (O'Halloran *et al.*, 2019, O'Halloran *et al.*, 2020); at least one person required medical treatment. Although all cats had signs of active disease with purulent draining lesions, it could not be confirmed that these positive test results resulted from transmission of *M. bovis* from the cats to the humans, from handling the food, or from other sources.

While purulent lesions have been identified as a route of transmission for mycobacteria from cats to humans (O'Connor *et al.*, 2019), respiratory routes of transmission should also be considered. While a productive cough is an uncommon clinical sign in cats with mycobacteriosis, if AFB have been identified

in BAL samples then obtaining this sample may have resulted in the generation of infectious aerosols which could pose a risk to humans (Human Animal Infections and Risk Surveillance (HAIRS) group, 2014).

While other species of mycobacteria that cause disease in cats, such as *M. microti*, *M. avium*, and *M. malmoense,* can cause disease in humans (Emmanuel *et al.*, 2007, Panteix *et al.*, 2010, Doig *et al.*, 2002, Russell *et al.*, 2014), none of these cases have been associated with a clinically sick cat. Therefore, the greatest risk posed from cats to humans regards feline infections with *M. bovis*, hence why it is important to accurately obtain a species-level diagnosis.

## 1.3 Mycobacterial Disease in Other Felids

While the term 'feline mycobacteriosis' is typically thought of as pertaining to disease in domestic cats, particularly in the UK where there are no indigenous wild felids other than the Scottish wildcat (*Felis silvestris silvestris*), it could be extended to mean mycobacterial infection within any member of the Felidae family. The Felidae family comprises 41 species (Kitchener *et al.*, 2017) and they inhabit a diverse range of habitats with near global distribution. However, both the range and number of wild felid species are declining, in part driven by an increasing and expanding human population, which has resulted in many felid species becoming at increased risk of extinction (Lamberski, 2015). In addition to the effect of human activities, infection and disease play an important part in shaping wild felid populations, and this includes infection with mycobacteria. A thorough understanding of mycobacterial infections within these populations is important to help protect and preserve these creatures, as they may play a role in the wider epidemiology of bTB (Renwick *et al.*, 2007), and being able to accurately diagnose mycobacterial infections prior to transportation of captive animals as part of conservation breeding programmes can help reduce the risk of introducing infection to naïve individuals (Molenaar *et al.*, 2020).

### 1.3.1 Species Affected

Mycobacterial infections have been reported in a wide range of Felidae species other than the domestic cat; this includes both wild and captive animals. Most reports concern mycobacteriosis in the lion (*Panthera leo)* (Keet *et al.*, 1996, Morris *et al.*, 1996, Keet *et al.*, 2010, Miller *et al.*, 2012, Miller *et al.*, 2015, Viljoen *et al.*, 2015, Sylvester *et al.*, 2017); this could reflect the close interaction of this species with buffalo, and this dynamic multi-species system of disease transmission has been investigated to try and understand what is happening, and determine what can be done to help protect animal populations (Keet *et al.*, 1996, Renwick *et al.*, 2007). The population genetics of lions are also thought to play a role in their seemingly increased susceptibility to mycobacterial infections, as it has been

suggested that inbreeding depression arising from isolated populations of lions is associated with an increased risk of mycobacteriosis compared to more outbred lions (Trinkel *et al.*, 2011). There are fewer documented cases of mycobacteriosis in other felids, namely cheetah (*Acinonyx jubatus*) (Keet *et al.*, 1996, Kerr *et al.*, 2020), tiger (*Panthera tigris*) (Van de Watering *et al.*, 1972, Lantos *et al.*, 2003, Cho *et al.*, 2006), snow leopard (*Panthera unica*) (Helman *et al.*, 1998), leopard (*Panthera pardus*) (Thorel *et al.*, 1998, Renwick *et al.*, 2007), Iberian lynx (*Lynx pardinus*) (Briones *et al.*, 2000, Pérez *et al.*, 2001, Aranaz *et al.*, 2004), bobcat (*Lynx rufus*) (Bruning-Fann *et al.*, 2001), clouded leopard (*Neofelis nebulosa*) (Cerveny *et al.*, 2013), jaguar (*Panthera onca*) (Kapustin *et al.*, 2006), cougar (*Puma concolor*) (Traversa *et al.*, 2009) and the European lynx (*Lynx lynx*) (Schmidbauer *et al.*, 2007, Kohl *et al.*, 2018). These species may reflect spillover hosts, with limited onward transmission or maintenance of infection within these populations.

## 1.3.2 Aetiology

Whereas many different species of mycobacteria have been identified as causing disease in domestic cats, nearly all culture or PCR-confirmed cases of mycobacteriosis in other felids are due to infection with *M. bovis* (Van de Watering *et al.*, 1972, Helman *et al.*, 1998, Thorel *et al.*, 1998, Briones *et al.*, 2000, Kirberger *et al.*, 2006, Keet *et al.*, 2010, Viljoen *et al.*, 2015, Sylvester *et al.*, 2017, Viljoen *et al.*, 2019). The dynamics of *M. bovis* infection in African wildlife populations has been explored, and it is thought most infections in lions arise from hunting and feeding on infected buffalo (Keet *et al.*, 1996, Renwick *et al.*, 2007). Studies into the molecular epidemiology of *M. bovis* infections of African wildlife species have identified that isolates across and within species have a high degree of genetic similarity, providing further evidence of the transmission of *M. bovis* in this ecosystem (Renwick *et al.*, 2007). This further confirms the lack of host specificity for *M. bovis* compared to other members of the MTBC, where infection and disease with pathogens such as *M. tuberculosis* is largely restricted to its specific host *i.e.*, humans. An infection with *M. caprae*, then designated *M. bovis* subsp. *caprae*, was reported in a captive tiger (Lantos *et al.*, 2003). To date, there have been no reports of *M. microti* infections in felids other than the domestic cat.

Reports of NTM causing disease in other felids are uncommon and appear restricted to animals in captivity. A diagnosis of disseminated *M. avium* infection was made on PCR in a captive Siberian tiger that was fed freshly culled chickens (Cho *et al.*, 2006); PCR testing on some of these chicken carcases were positive for *M. avium* despite showing no visible lesions. Disease due to RGM has also been identified, with *M. fortuitum* isolated from a clouded leopard (Cerveny *et al.*, 2013). Isolation of NTM has also been reported in animals without evidence of clinical disease (Traversa *et al.*, 2009), as well as cases of *M. bovis*-NTM co-infection (Miller *et al.*, 2019). It is thought that some NTM infections could arise from contamination of wounds that arise through hunting and other aggressive behaviours (Miller *et al.*, 2019).

### 1.3.3 Clinical Signs

Across non-domestic cat felid species, the clinical signs associated with mycobacterial infection are broadly similar. Disease is typically more advanced in free-ranging felids compared to captive animals, and some wild felids may be found dead (Keet *et al.*, 1996, Peña *et al.*, 2006). Like domestic cats, non-specific signs of ill health such as weight loss, anorexia, and lethargy are commonly reported in other felid species (Keet *et al.*, 1996, Morris *et al.*, 1996, Cho *et al.*, 2006, Schmidbauer *et al.*, 2007, Cerveny *et al.*, 2013). Evidence of respiratory compromise is also frequent, with signs such as coughing, dyspnoea and tachypnoea identified (Morris *et al.*, 1996, Helman *et al.*, 1998, Lantos *et al.*, 2003); cutaneous lesions akin to those seen in domestic cats are less common (Cerveny *et al.*, 2013), but lesions such as elbow hygromas have been described (Kirberger *et al.*, 2006). Lameness and associated signs such as joint thickening and reduced flexion are not uncommon among felids (Van de Watering *et al.*, 1972, Briones *et al.*, 2000, Kapustin *et al.*, 2006), and there have also been reports of ocular disease resulting in corneal opacity (Keet *et al.*, 1996) and blindness (Peña *et al.*, 2006).

### 1.3.4 Diagnostic Testing

As with domestic cats, the *ante-mortem* diagnosis of mycobacteriosis in other felids is challenging, but it has been at the centre of increased research over the last decade. An obvious challenge for testing in these species is obtaining samples safely, minimising risk to both the animal and those involved in sample collection.

Unlike in domestic cats where TST is not recommended, this is a test often performed to facilitate in the diagnosis of mycobacterial infections in other felids. Out of 52 lions with a culture-confirmed diagnosis of *M. bovis*, 51 (98.1%) responded to PPDB on TST, with 45 (86.5%) defined as TB-reactors where the response to PPDB exceeded that to PPDA by at least 2mm (Keet *et al.*, 2010); those that were not defined as TB-reactors may have been missed due to co-infection with NTM. It was also shown that co-infection with FIV did not have a negative effect on the size of cutaneous reactions in TST, which may be meaningful in the application of other tests of CMI to felids, such as the IGRA. Other studies have also identified a substantial number of felids responding positively on TST (Miller *et al.*, 2012, Kerr *et al.*, 2020, Molenaar *et al.*, 2020), although this is not consistent across all reports (Van de Watering *et al.*, 1972, Miller *et al.*, 2019). The performance of the TST in lions has been subject to review (Viljoen *et al.*, 2019), suggesting differences in the response of wild *vs.* captive lions, with captive animals showing a higher mean response to PPDA compared to wild lions across different locations. As for cattle, it was recommended that a comparative TST should be employed to account for infection with or exposure to NTM, and that species-specific cut-off values should also be used to maximise performance of the test (Viljoen *et al.*, 2019). Additionally, TST requires the animal to be held or

recaptured and immobilised after three days to assess the response, making it logistically challenging to perform this test.

Given that the IGRA was successfully adapted for use in domestic cats, a lion-specific IGRA was subsequently developed (Maas *et al.*, 2012). The methodology of the lion-specific IGRA is almost identical to that of the cat IGRA, however whole blood is used compared to isolating PBMCs, which makes this assay a more attractive format for use in the field. This study generated monoclonal anti-lion IFNγ antibodies, which could detect IFNγ from tigers, domestic cats and the maned wolf (*Chrysocyon brachyurus*) but use of this assay in a diagnostic capacity for mycobacterial infections in lions, or other species, has not been reported. In contrast, the cat-specific IGRA has been used in lions as part of pre-movement screening (Molenaar *et al.*, 2020); positive results suggestive of *M. bovis* infection were reported, while PCR on peripheral blood supported a diagnosis of infection with a member of the MTBC. This may suggest clinical utility of the cat-specific IGRA for lions and potentially other felids, although this requires further validation.

Tests of humoral immunity, which are frequently employed for wildlife species (Lyashchenko *et al.*, 2008), have also been used in felids. This includes use of rapid tests for the detection of antibodies against combinations of MPB83, ESAT-6, CFP-10, and their fusion protein (ESAT-6/CFP-10) (Miller *et al.*, 2012, Kerr *et al.*, 2020). Across two rapid test platforms, 58-80% of *M. bovis*-positive lions were antibody-positive (Miller *et al.*, 2012, Miller *et al.*, 2019), which is consistent with reasonable test performance data for *M. bovis*-infected domestic cats (Rhodes *et al.*, 2011). Antibody-positive responses on rapid tests or MAPIA have also been reported in a cheetah (Kerr *et al.*, 2020) and a jaguar (Kapustin *et al.*, 2006). Rapid tests can be used as a point-of-care diagnostic, allowing for decision making once a test result is obtained without having to recapture animals at later date as for TST, but there remain concerns about the sensitivity of antibody-based diagnostics in recently infected animals.

An area of research which has gathered more attention for the diagnosis of mycobacteriosis in other felids is that of GEAs. A GEA for CXCL9, a potent chemokine for the chemotaxis, differentiation, and multiplication of lymphocytes, has been developed for use in lions (Olivier *et al.*, 2017, Sylvester *et al.*, 2017), and successfully tested on a cheetah (Kerr *et al.*, 2020). In this assay, RNA is extracted from a heparinised whole blood sample following incubation with mycobacterial antigens (ESAT-6/CFP-10, and TB7.7). Quantitative PCR for reverse transcribed copy DNA from the extracted RNA is then performed; for lions it was shown that detection of CXCL9 mRNA was the most sensitive measure for *M. bovis* infection relative to the *yhwaz* housekeeping gene (Olivier *et al.*, 2017). This GEA showed similar results of *M. bovis* prevalence in lions compared to TST (Sylvester *et al.*, 2017, Viljoen *et al.*, 2019), making it an attractive alternative, although it requires laboratory access for the assay to be performed.

Samples may also be taken *ante-mortem* for culture, PCR testing, cytology, or histopathology with special staining or, more frequently, these samples will be obtained at *post-mortem* examination. Histopathological changes associated with mycobacterial infections in these species mirror those

reported in domestic cats, with granulomatous to pyogranulomatous inflammation, dominated by epithelioid macrophages, variable amounts of necrosis, lacking mineralisation, and with rare MNGCs (Van de Watering *et al.*, 1972, Keet *et al.*, 1996, Morris *et al.*, 1996, Helman *et al.*, 1998, Lantos *et al.*, 2003, Cho *et al.*, 2006, Peña *et al.*, 2006, Kapustin *et al.*, 2006, Cerveny *et al.*, 2013, Kerr *et al.*, 2020). Special staining with ZN, FF, IHC or fluorescence microscopy may reveal no to abundant mycobacteria (Van de Watering *et al.*, 1972, Helman *et al.*, 1998, Bruning-Fann *et al.*, 2001, Pérez *et al.*, 2001, Peña *et al.*, 2006, Cerveny *et al.*, 2013). Samples obtained via BAL have identified the presence of mycobacteria (Miller *et al.*, 2015), which could indicate that the respiratory route is a means of transmission of *M. bovis* in these species to other animals; however, it could also have important implications for veterinary staff dealing with such cases given this is a significant route for human infection. This is supported by finding cavitary pulmonic lesions in felids at *post-mortem* (Van de Watering *et al.*, 1972, Keet *et al.*, 1996, Helman *et al.*, 1998).

Non-specific diagnostics may be performed, as for domestic cats, particularly screening for infection with FIV (Peña *et al.*, 2006, Keet *et al.*, 2010, Cerveny *et al.*, 2013); while a high proportion of lions that are infected with *M. bovis* are also FIV positive (Keet *et al.*, 1996, Keet *et al.*, 2010, Sylvester *et al.*, 2017) there is insufficient evidence to state that there is immunosuppression resulting from FIV infection, contributing to infection with *M. bovis* or the development of tuberculous disease. Haematological and serum biochemical findings in cases of mycobacteriosis in other felids are poorly described; leukocytosis has been reported in one tiger (Van de Watering *et al.*, 1972), leukocytosis, hyperglobulinaemia, hypoalbuminaemia, and hypercalcaemia have been reported in a lion (Morris *et al.*, 1996), and anaemia, azotaemia, hyperphosphataemia, hypoalbuminaemia and hyperglobulinaemia were identified in a clouded leopard (Cerveny *et al.*, 2013). These findings are broadly in-line with what is reported in cats, and likely reflect a combination of inflammatory and infectious processes and are non-specific for mycobacterial infection, although hypercalcaemia would result in consideration of granulomatous inflammation as a cause of this change. Imaging has been infrequently performed, but on thoracic radiography cavitary lesions have been reported (Van de Watering *et al.*, 1972, Helman *et al.*, 1998), sometimes in addition to a bronchointerstitial pattern (Morris *et al.*, 1996, Lantos *et al.*, 2003). These cavitary lesions are believed to be consistent with an inhaled route of infection, which differs from the proposed route of infection of domestic cats. Radiographic changes of bone and joint lesions have also been described, with a mixed pattern of proliferative periosteal reactions and subchondral bone sclerosis and lysis (Van de Watering *et al.*, 1972, Kirberger *et al.*, 2006).

### 1.3.5 Treatment and Prognosis

Unlike in domestic cats, cases of mycobacteriosis in other felids are generally not treated. It is not feasible to treat free-ranging wildlife, and while attempts have been made to treat captive animals (Van de Watering *et al.*, 1972, Cho *et al.*, 2006) this has not been successful. Treatment failure in one tiger was due to the use of agents that would not be effective against mycobacteria (Cho *et al.*, 2006),

whereas the other tiger did receive drugs effective against mycobacteria *e.g.,* para-amino salicylic acid (Zhang *et al.*, 2019), but it eventually succumbed to infection (Van de Watering *et al.*, 1972). In other cases of MTBC infection in captive animals, the risk of progression to clinical disease and subsequent transmission to other animals and potential humans was deemed too great, resulting in euthanasia (Molenaar *et al.*, 2020).

## 1.4 Aims and Objectives

The aim of this project is to improve current diagnostics techniques and develop new assays for the rapid and accurate identification of cases of feline mycobacteriosis, and in particular feline TB infections.

This will be achieved through the following objectives:

1. To determine whether histopathological features and inflammatory cell populations present in feline mycobacterial skin and lymph nodes lesions are suggestive of infection with either *M. bovis* or *M. microti*, as well as describing the lesions in cases of a less frequent presentation of mycobacterial disease, ocular mycobacteriosis, facilitating more rapid diagnosis.
2. To critically evaluate the performance of the IGRA and determine whether changes to antigen positivity cut-off thresholds could improve sensitivity and specificity, and update reporting guidelines for results of this test, as well as identify whether serial IGRA testing is of benefit for monitoring the response to treatment.
3. To assess whether the feline IGRA could be of use for the diagnosis of mycobacteriosis in other felids through computational analysis of IFNγ sequence data and determining anti-cat IFNγ antibody cross-reactive with recombinant IFNγ (rIFNγ) proteins from other Felidae.
4. To develop novel diagnostic tests, namely an ELISA for the detection of antibodies specific for the diagnosis of TB, providing an assessment of the feline humoral response to TB infections which is currently unavailable, and a qPCR optimised for use on FFPE tissues specifically targeted against the main mycobacterial pathogens of cats.

# Chapter 2: Ocular Mycobacteriosis: Comparative Aspects, and Histopathological and Immunohistochemical Characterisation of Feline Ocular Lesions

## Preface

The following chapter consists of one article published in Ocular Immunology and Inflammation. Copyright of the published article is assigned to Taylor & Francis. Permission was granted to reproduce the Author Accepted Manuscript on the condition that the original source of publication is acknowledged.

**Mitchell, J. L.,** Ganis, L, Blacklock, B. T., Petrushkin, H., Hope, J. C. and Gunn-Moore, D. A. Ocular Tuberculosis: More than 'Of Mice and Men'. *Ocular Immunology and Inflammation*, 2020; 1-5.

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This chapter also includes a reformatted version of a manuscript which has been revised and re-submitted to Veterinary Pathology.

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## Author's Contribution

The author performed the literature review, took the histopathological image, and wrote the manuscript for the article "Ocular Tuberculosis: More than 'Of Mice and Men'". The author selected cases, performed immunohistochemical staining, analysed the data and wrote the manuscript for the article "Ocular Mycobacterial Lesions in Cats". Staining with ZN was performed by Laura MacDougall (Royal (Dick) School of Veterinary Studies and the Royal Veterinary College), and H&E and Masson's Trichrome (MT) staining were performed by the Veterinary Pathology Unit (VPU) (Royal (Dick) School of Veterinary Studies).

## 2.1 Introduction

Mycobacterial infections in cats have been documented for over 100 years (Jensen, 1891), but the recognition of feline mycobacteriosis had declined due to fewer reported cases, in part driven by practices such as the pasteurisation of milk (Snider, 1971, Snider *et al.*, 1971). Consequently, mycobacteriosis may have not been considered as a differential diagnosis for cases of disease in cats. In GB, there has been increasing awareness and reporting of these infections since the mid-1990's (Gunn-Moore *et al.*, 1996) and now mycobacterial infection is more likely to be considered in cats presenting with ulcerative and/or discharging cutaneous lesions, as this is the most common presentation of feline mycobacterial disease (Gunn-Moore *et al.*, 2011a). However, not all cases present with the typical clinical signs of cutaneous lesions with or without a local lymphadenopathy. One such 'unusual' presentation of feline mycobacteriosis is that of ocular disease (Stavinohova *et al.*, 2019).

Studies into human ocular mycobacteriosis have focused on cases of OTB, describing the clinical presentations of such infections, the immunopathogenesis, diagnostic approach and treatment. There has been recent renewed interest in OTB, with collaborative groups aiming to provide consensus data on how to approach these infections to optimise and improve patient outcomes (Petrushkin *et al.*, 2020, Agrawal *et al.*, 2021). However, to expand our knowledge further on this subject would require the use of models, animal or cellular, to fully interrogate the underlying pathogenesis of OTB (Basu *et al.*, 2020). Animal models of OTB have relied on experimental infections of laboratory species to induce ocular lesions, but these may not accurately reflect naturally occurring cases of human OTB due to differences in the host immune response, the infecting organism, the route of infection, or the infective dose (Woods *et al.*, 1958, Rao *et al.*, 2009, Takaki *et al.*, 2018). Therefore, spontaneous natural animal models of OTB would be of interest to ascertain the similarities and differences with human OTB as these may prove more suitable for the study and understanding of such infections in humans. Given the fact that naturally occurring ocular mycobacteriosis is reported in cats (Dietrich *et al.*, 2003, Fyfe *et al.*, 2008, Stavinohova *et al.*, 2019), it would be prudent to explore and understand this disease entity in more detail to determine whether cats could act as a suitable model for human OTB.

Ocular disease is reported in nearly 7% of domestic cats attending primary veterinary care in England (O'Neill *et al.*, 2014), where it may be part of a systemic disease process or localised to the ocular, periocular and orbital structures. Infectious causes of feline ophthalmic disease include toxoplasmosis (Davidson and English, 1998), feline infectious peritonitis (FIP) (Pedersen, 2009, Kipar and Meli, 2014), and mycobacteriosis, among many others (Aroch *et al.*, 2008). Mycobacterial infection as a cause of ocular disease is less well appreciated, despite historical reports (Lawford and Neame, 1923). A recent study showed that approximately 6% of cats with mycobacteriosis present with ocular lesions (Gunn-Moore *et al.*, 2011a), which may be seen in association with systemic disease (Stavinohova *et al.*, 2019). Clinical signs associated with feline ocular mycobacteriosis vary depending on the tissue(s) affected and route of infection, ranging from corneal or conjunctival granulomas (Deykin *et al.*, 1996, Lamagna *et al.*, 2009, O'Brien *et al.*, 2017b) to uveitis (Stavinohova *et al.*, 2019), and even acute onset

blindness (Formston, 1994, Stavinohova *et al.*, 2019). Both MTBC and NTM can cause ocular disease in cats (McIntosh, 1982, Formston, 1994, Deykin *et al.*, 1996, Dietrich *et al.*, 2003, Davies *et al.*, 2006, Gow, 2006, Fyfe *et al.*, 2008, Lamagna *et al.*, 2009, O'Brien *et al.*, 2017b, Stavinohova *et al.*, 2019), and treatment typically consists of a combination of surgery (*i.e.,* enucleation) and systemic antimycobacterial therapy, with remission rates of 80% having been reported (Stavinohova *et al.*, 2019).

The first step towards diagnosing a case of ocular mycobacteriosis in a cat is through histopathological examination of an enucleated globe in conjunction with the adnexal and supporting connective tissues. Unlike in humans, where cases of OTB are treated with the eye left *in situ* (Agrawal *et al.*, 2017), enucleation is the mainstay of both diagnosis and treatment of feline ocular mycobacterial infections (Stavinohova *et al.*, 2019), in part because it is assumed that an ocular lesion is neoplastic rather than due to an infectious or inflammatory process. This provides a resource for the immunopathological characterisation of feline ocular mycobacterial lesions, but detailed descriptions of the features associated with these lesions are lacking. Most histopathological and immunological studies into feline mycobacteriosis have focused on the more commonly associated cutaneous and lymph node lesions (Kipar *et al.*, 2003, Peterhans *et al.*, 2020), as also described in Chapter 3 (Mitchell *et al.*, 2021b). However, understanding the changes taking place within the orbital and periocular structures in cases of ocular mycobacteriosis can assist with the diagnosis of such infections, as there are other pathogens that can cause pyogranulomatous ocular lesions in cats (Ziółkowska *et al.*, 2017, Nunes Rodrigues *et al.*, 2020). This would allow for comparisons between the features of mycobacterial lesions at different sites in the body, namely cutaneous and lymph node lesions, to determine whether there are specific features of ocular lesions that may result from it being an immunoprivileged site. For example, cases of ocular FIP can present with more B-cells than seen in lesions in other sites (Ziółkowska *et al.*, 2017), which may suggest differences in the local ocular immune response.

The aims of this chapter, presented in the following paper and submitted manuscript, were to compare the presentations of OTB in cats and humans to determine what similarities or differences exist, as well as to make comparisons with other species that may serve as useful animal models for OTB such as cattle. Secondly, the histopathological features and cell populations of ocular mycobacterial lesions in cats were investigated with routine and immunohistochemical staining; the distribution of these cell types was also explored throughout the ocular and periocular tissues as this may provide further insight into the potential routes of ocular infection. Ziehl-Neelsen staining was performed to identify mycobacteria within ocular lesions, and these were scored on a modified Bacillary Index (BI; described further in Chapter 3); the presence of mycobacteria within the eye would infer that infection drives the inflammatory response, rather than it resulting from antigenic stimulation from a distant site elsewhere in the body. This would serve to not only facilitate the diagnosis and understanding of feline ocular mycobacterial disease, but also to provide further evidence as to whether the cat may or may not be a suitable model for human OTB infections.

# Ocular tuberculosis: More than ‘Of Mice and Men’

**Abstract**

Tuberculosis (TB), caused by infection with members of the *Mycobacterium tuberculosis*-complex, is one of the oldest known infectious disease entities, resulting in the death of millions of humans each year. It also results in a substantial degree of morbidity and mortality in animal species. Extrapulmonary TB is well-recognised in humans, and the eye is one site that can be affected. Studies seeking to understand ocular TB have often relied on animal models; however, these have their limitations and may not truly reflect what happens in humans. We wish to raise awareness among ophthalmologists and vision scientists of naturally occurring cases of ocular TB in animals, namely cattle and domestic cats, and the possibilities of gaining further understanding of this presentation of TB by adopting a collaborative approach. This will hopefully improve outcomes for both human and animal patients.

**Keywords:** ocular, tuberculosis, zoonosis, uveitis, feline, bovine

## Ocular tuberculosis: More than ‘Of Mice and Men’

Tuberculosis (TB), predominantly caused by *Mycobacterium* (*M.*) *tuberculosis*, remains the largest single infectious cause of death in humans, with 10 million individuals becoming ill with TB in 2018 and a reported 1.5 million TB-related deaths.[1] Although predominantly an infection of the lungs, extrapulmonary TB accounted for 15% of the 7 million incident cases of TB recorded in 2018;[1] one site that can become affected is the eye. Ocular TB (OTB) is thought to originate from haematogenous dissemination of bacteria from a site of primary infection *i.e.* the lungs to the eye,[2] and while every ocular tissue can become affected,[3] choroidal tubercles are the most common presentation of disease.[4]

There has been renewed interest in animal models of OTB, and the advantages and limitations of these have recently been reviewed.[5] Animal models allow us to ask questions that would not be possible in human studies; however, these host-pathogen interactions could be deemed artificial, they may not truly reflect the progression of disease in humans, and the ethics of using experimental research animals should be considered. Progress has been made in the use of *in vitro* models, although further development is required to allow complete exploration of such a complex structure.[6] Following the review by Basu *et al.*,[5] we propose that naturally occurring cases of OTB in animals may provide more meaningful results than current animal models, and through this communication we seek to raise awareness of these infections; human and veterinary medicine benefit when they work alongside one another, and a shared understanding and interest in this field could greatly assist all patients, whether they ambulate on two limbs or four.

While TB refers to infection with members of the *M. tuberculosis*-complex,[7, 8] non-tuberculous mycobacteria can also cause ocular disease in humans[9, 10] and animals.[11-15] Reports of OTB in animals are often limited to small case studies, resulting in gaps in our knowledge of the dynamics of these infections, as well as species-to-species variation. Of the species susceptible to developing TB, cattle are the obvious animal to investigate further, given the high prevalence of bovine TB (bTB) in parts of the world such as the United Kingdom and the Republic of Ireland.[16] The most common cause of bTB is *M. bovis*, which can also cause disease in humans;[1] resultantly, the term "zoonotic TB" has been used for *M. bovis* infections in humans.[17] One of the benefits of using cattle to study OTB is the highly homologous immunopathology between bTB and human TB.[18, 19] While reports of OTB in cattle are rare,[20-22] preliminary studies by the authors have shown ocular signs present in a number of eyes taken from animals infected with *M. bovis* (Figure 1) (unpublished data). Clinically, ocular bTB presents similarly to disease in humans, with silent choroidal granulomas which can result in a subretinal exudate and retinal detachment.[20, 21] Disease is thought to originate from haematogenous dissemination of bacteria from the lungs, mirroring the proposed route of human ocular infection. Anterior uveitis and keratitis have also been reported.[20, 22] Further work is required to establish the prevalence of ocular bTB, as well as provide more detailed descriptions of the macrophenotypic presentations of disease, but cattle may prove a useful animal model in the future.

An often-overlooked species to investigate further would be the domestic cat. Once considered a historical disease associated with the consumption of raw milk from tuberculous cows, feline TB has been increasingly recognised in Great Britain in recent years,[23, 24] and is of importance because of the potential zoonotic risk.[25] Feline TB is caused by infection with either *M. bovis* or the vole bacillus, *M. microti*;[23] of note, cats appear highly resistant to infection with *M. tuberculosis.*[26] Feline TB typically presents as a nodular cutaneous disease; pulmonic involvement is putatively due to haematogenous spread of bacteria from the site of primary infection.[27] Genetically *M. microti* is very similar to *M. bovis*-BCG,[28] and historically it was used in TB vaccinations for humans as it had been assumed to be avirulent,[29] as it lacks key virulence factors encoded on the region of difference 1 (RD-1) locus of the genome.[30, 31] These RD-1 factors have traditionally been thought of as key for mycobacterial virulence, yet the extent of disease due to *M. microti* in the domestic cat,[32-34] and other species such as dogs,[35] goats,[36] meerkats,[37] alpaca and badgers,[29] shows this pathogen can have devastating consequences. Additionally, a small number of cases of *M. microti* TB have also been recorded in humans.[38]

In Great Britain, just over 6% of cats with mycobacterial disease present with ocular signs,[23] which can result from infection with both tuberculous and non-tuberculous mycobacteria.[39-44] Unlike in humans and cattle, cases of feline OTB typically present as clinically fulminant disease with active lesions,[39, 44] and the consequences of untreated disease can be devastating. Feline OTB has been recognised since the early 20th century, with reports featuring in prominent medical ophthalmology journals,[45] and there has been recent renewed interest in this disease entity.[44] Most cases of OTB appear to result from *M. bovis* infection, though *M. microti*-associated disease has also been identified.[44] Cases often present with signs attributable to uveitis, which can vary from localised findings including scleral injection, corneal oedema, aqueous flare and a swollen iris to subtle changes associated with pain, namely

hyporexia and lethargy;[44] these changes may be subtle and not readily appreciated. Fundoscopic examination typically identifies solitary choroidal tubercles or tuberculomas (Figure 2).[44] Retinal detachment often accompanies choroidal lesions, with an associated subretinal exudate and haemorrhage; these cats will typically present with fixed, dilated pupils and are non-visual.[39, 44, 45] Secondary complications of uncontrolled uveitis, such as cataract formation and glaucoma, have also been recorded.[39, 44] Other phenotypes seen in humans, such as serpiginous-like choroiditis and retinal vasculitis have not been documented in cats with OTB; although retinal vasculitis is a classical finding in cats with ocular manifestations of feline infectious peritonitis.[46] Corneal and conjunctival granulomas have been recorded in cats,[44] but lesions affecting tissues other than the uvea or retina are less common. Histologically, granulomatous to pyogranulomatous inflammation is the dominant finding in feline OTB lesions,[44] however multinucleated giant cells, a hallmark of tuberculous lesions in other species, including those affecting ocular tissues,[47] are rare in cats.[44, 48] Cases of feline OTB may present with or without systemic disease.[39, 44, 49]

Of the other species susceptible to TB, naturally occurring disease is only frequently identified in South American camelids (SAC),[50] lions[51] and badgers;[52, 53] while reports of OTB in SAC are lacking, the authors are aware of possible cases in the United Kingdom and investigations into these are ongoing (Dr S. J. Moore, personal communication, 18 June 2020). The pathology of OTB in lions appears similar to domestic cats, with reports of *M. bovis* causing granulomatous uveitis and subsequent retinal detachment.[54] As for SAC, OTB has not been reported in badgers. Cases of canine TB are uncommon, and dogs appear to be more resistant to mycobacterial infections compared to cats, but they are susceptible to *M. tuberculosis*,[55] posing a risk to human health.[56] Ocular involvement in canine TB is rare, with lesions most often present within the choroid.[57] Psittacines can also develop disease due to *M. tuberculosis* infection, where it can result in retro-orbital infection[58] or tubercles on the nictitating membrane as part of a disseminated disease process.[59] Disseminated disease resulting in OTB has also been observed in pigs[21] and non-human primates;[60] sometimes ocular signs may be the only observed clinical finding. Determining the extent of disease is essential for the appropriate treatment of both human and animal cases.[2, 44, 61]

The exact pathogenesis of ocular involvement in TB in animal species is unknown; it could mirror human infection, with haematogenous seeding of bacteria from a primary site of infection to the eye, it may result from direct ocular injury or it could be a sterile inflammatory response to infection elsewhere in the body. Our ongoing studies are exploring the histopathology and immunology of feline TB lesions to expand our knowledge of host-pathogen interactions, the extent to which ocular structures are affected (Figure 3), and whether infectious agents are present within the eye. Antigenic mimicry between *M. bovis*-BCG and retinal antigens may be the cause of uveitis, chorioretinitis, other retinopathies and optic neuropathy in some humans;[62-66] it is unknown whether this phenomenon occurs in other species. The difference between knowing whether the eye contains infectious organisms, or if the clinical signs signify local hypersensitivity, could influence the management of OTB across all species.[65, 66] While most cases of human OTB are presumptive, diagnosed with the eye *in situ* and treated with systemic anti-mycobacterial therapy,[2, 61] the diagnosis of feline OTB is often achieved on histopathology of the

enucleated globe. Anti-mycobacterial treatment is then given to target any residual, or systemic infection. If such cases can be successfully identified and treated without requiring enucleation, as achieved with humans, this may provide a more positive outcome for cats and their owners. For human ophthalmologists, these findings could identify more appropriate, spontaneous models to study the pathology of OTB. They could also facilitate diagnosing these infections,[67] as well as inform whether prolonged courses of systemic anti-mycobacterial therapy are necessary for successfully treating OTB or whether treating the inflammatory component of the disease is sufficient if the pathology is not driven by active infection.[65, 66]

Ocular TB should not be thought of as solely a human disease; while not identical, this condition is also recognised in many species including cattle and cats. The underlying immunopathology of TB is similar between humans and cattle, and this species may provide more beneficial insights to OTB than the laboratory animal species currently used. While the feline immune response to mycobacterial infection differs from that of humans,[48] comparative studies can be undertaken to ask what similarities and differences are observed, why this happens and subsequently what can be done to improve outcomes for both species. The best way to combat these zoonotic infections is not to divide knowledge between the species, but to share our collective understanding for the benefit of all.

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**Figure Captions**

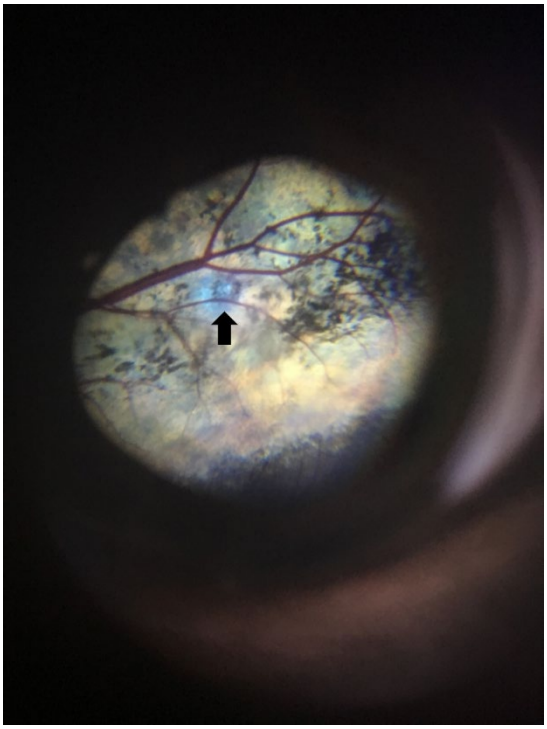

Figure 1: Fundoscopic examination of the eye from a cow with confirmed *Mycobacterium bovis* infection showing a choroidal granuloma (black arrow).

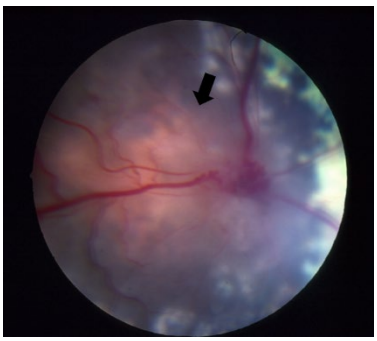

Figure 2: Fundoscopic examination of the eye of a cat with suspected tuberculosis, showing a choroidal granuloma (black arrow) within the region of the non-tapetal fundus. (Image courtesy of David Gould MRCVS).

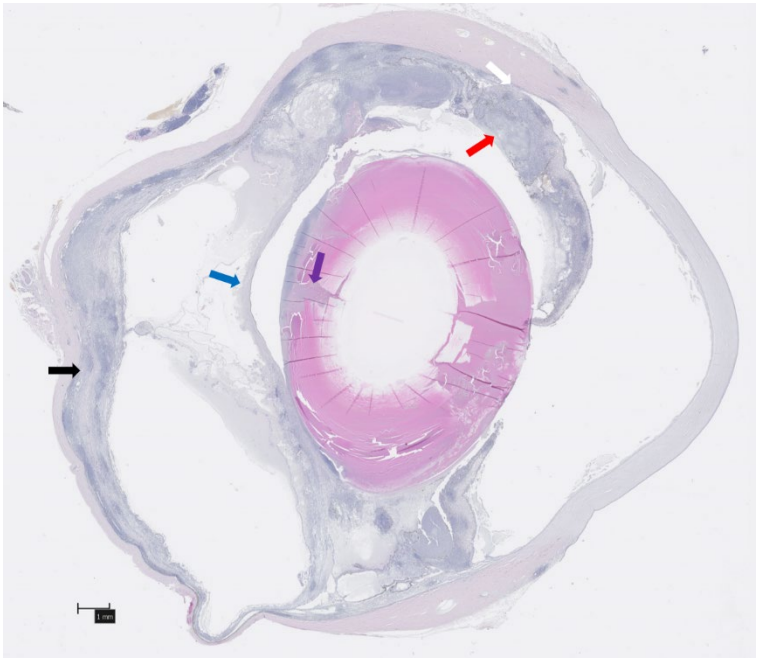

Figure 3: Haematoxylin and eosin stained section of the globe from a cat diagnosed with *Mycobacterium bovis* by interferon-gamma release assay testing[68]. There is evidence of granulomatous to pyogranulomatous chorioretinitis (black arrow) extending into the anterior uvea (red arrow); there is also blockage of the drainage angle (white arrow). The retina is detached (blue arrow) and there is posterior rupture of the lens capsule (purple arrow), with subsequent neutrophilic phakitis. Scale bar = 1mm. Magnification x18.

## 2.2 Materials and Methods

### 2.2.1 Animals and Samples

Ethical approval for this study was granted by The University of Edinburgh Veterinary Ethical Review Committee (approval no. 79 14).

Formalin-fixed paraffin-embedded globes with a morphological diagnosis consistent with (peri)ocular mycobacteriosis *i.e.,* a granulomatous or pyogranulomatous inflammatory infiltrate, predominantly comprised of epithelioid macrophages, with or without necrosis, were provided by board certified pathologists at Cytopath, Herefordshire, UK and Finn Pathologists, Norfolk, UK. Additional tissues were held at The Roslin Institute at The University of Edinburgh, Scotland. The globes had been previously enucleated by licensed veterinarians as part of *ante-mortem* diagnostic investigation and management of ocular disease, or they were taken at *post-mortem* examination. Where available, clinical records were provided following owner consent, and data were stored securely in accordance with data protection guidelines.

Samples were included if they showed well-preserved ocular structures and had histopathological evidence of granulomatous to pyogranulomatous lesions typical of mycobacterial disease. Ziehl-Neelsen negative samples were included if there was additional supporting evidence for mycobacterial disease, namely demonstration of AFB in non-ocular mycobacterial lesions from the same cat, a positive result on specialist mycobacterial culture, PCR, or IGRA (Rhodes *et al.*, 2008b), or if the board certified pathologist considered the histopathological appearance of the lesion(s) inconsistent with that seen with FIP or other causes of ocular (pyo)granulomatous inflammation. Where available, signalment data, the results of FeLV antigen and FIV antibody testing, and other clinical examination findings were recorded.

Control FFPE tissues for IHC were a cutaneous lesion from a cat diagnosed with MTBC infection positive for calprotectin by IHC (see Chapter 3) (Mitchell *et al.*, 2021b), the parietal lobe from a cat with cognitive dysfunction, positive for ionised calcium-binding adaptor protein-1 (Iba1), and a feline cutaneous non-epitheliotropic B-cell lymphoma with sparse non-neoplastic T-cells. Calprotectin is expressed by granulocytes, monocytes and recently blood-derived macrophages and granulocytes (Rugtveit *et al.*, 1996), Iba1 by macrophages and microglia (Imai *et al.*, 1996), CD3 by T-cells (Kipar *et al.*, 2003), and Pax5 by B-cells other than plasma cells (Agostinelli *et al.*, 2010).

## 2.2.2 Histopathology

Histopathology was performed to identify which ocular tissues contained inflammatory lesions, to describe the nature of the inflammatory cell population and score the intensity of inflammation based on the number of cells present. Four-micron thick sections were cut, mounted, and stained with H&E, ZN and MT. Slides were scanned using a NanoZoomer-XR scanner, using the NDP.scan Ver.3.2.12 software (Hamamatsu Photonics, Hamamatsu City, Japan) and images viewed on NDP.view2 Ver 2.7.52 (Hamamatsu Photonics). Sections stained with H&E were examined for the presence and population of inflammatory cells, the granuloma type *i.e.,* 'organised' (singular or multifocal zones of central caseous necrosis, surrounded by macrophages and neutrophils, with peripherally located lymphocytes and plasma cells) or 'atypical' (well defined clusters of macrophages and epithelioid macrophages divided by thin fibrous septa) (see Chapter 3) (Mitchell *et al.*, 2021b), and additional features, such as necrosis. The presence of collagen, indicating fibrosis, was assessed using the MT-stained slides. The inflammatory cell population was visually assessed and categorised as either: (i) (pyo)granulomatous if the dominant inflammatory phenotype consisted of macrophages and epithelioid macrophages, with variable numbers of neutrophils and fewer lymphocytes or plasma cells, (ii) lymphoplasmacytic or (iii) mixed if both (pyo)granulomatous and lymphoplasmacytic cells were present in similar numbers, or if the pattern of inflammatory changes identified segmental regions of infiltration with either cell population within the same tissue. The degree of inflammation was scored by counting the number of inflammatory cells present within each lesion using the cell detection feature in QuPath Ver 0.1.2 (Bankhead *et al.*, 2017); and assigned an inflammation score (Table 2.1). Slides stained with ZN were examined under light microscopy and the BI for tissues with inflammatory lesions was calculated as described in Chapter 3 (Mitchell *et al.*, 2021b), and a BI score and grade assigned (Table 2.2).

| **Inflammation Score** | **Number of Inflammatory Cells** |
|---|---|
| 0 | 0 |
| 1 (minimal) | Less than 1,000 |
| 2 (mild) | 1,000 – 10,000 |
| 3 (moderate) | 10,001 – 50,000 |
| 4 (marked) | 50,001 – 100,000 |
| 5 (extensive) | More than 100,000 |

**Table 2.1:** Inflammation scoring system used to assign grades for the number of inflammatory cells present within ocular lesions.

| BI Score | BI Grade | Average number of AFB/hpf |
|---|---|---|
| 0 | Low | 0 |
| 1 | Low | 0.01 – 0.1 |
| 2 | Low | 0.1 – 1 |
| 3 | High | 1 – 10 |
| 4 | High | 10 – 100 |
| 5 | High | 100 – 1000 |
| 6 | High | Over 1000 |

**Table 2.2:** BI scoring and grading system used to describe the average number of AFB present over 15 high power fields (hpf), adapted from the Ridley scoring system used in cases of leprosy in humans.

## 2.2.3 Immunohistochemistry

Expression of calprotectin, Iba1, CD3 and Pax5 was detected using IHC. Briefly, sections were mounted on SuperFrost® Plus-coated slides, dewaxed, rehydrated, rinsed in distilled water and washed in Tris-buffered saline (TBS) with Tween®20 (TBS-T). Antibodies were diluted in TBS-T and all washes were in TBS-T. Antigen retrieval followed by incubation with primary antibodies for 30 minutes at room temperature (RT) was performed as per Table 2.3. To block for non-specific endogenous peroxidase activity, REAL Peroxidase-Blocking Solution (Dako, Glostrup, Denmark) was used for 10 minutes at RT, before incubating sections with goat anti-mouse/anti-rabbit secondary detection polymer (EnVision™+ Dual Link System-HRP, Dako) for 40 minutes at RT. Positive staining was visualised using Liquid DAB+ Substrate Chromogen System (Dako) for 10 minutes at RT. Slides were counterstained with haematoxylin and Scott's Tap Water for 10 seconds each, dehydrated, cleared in xylene, mounted and a cover-slip applied.

A concentration-matched isotype control was used to assess non-specific staining (mouse immunoglobulin [Ig]G1 antibody, MCA928, BioRad, Hercules, California, USA). Negative controls were run with omission of the primary antibody. Immunolabelled slides were scanned as previously described and examined to determine the relative degree of positive staining for each IHC marker, and the distribution of positive cells within the lesion(s).

| Target | Antibody | Source | Antigen retrieval | Primary antibody concentration or dilution |
|---|---|---|---|---|
| Calprotectin | Mouse monoclonal anti-human macrophages, clone MAC387 (MCA874G) | BioRad | Proteinase K (Dako)<br><br>20 minutes at RT | 1:800 (1.25µg/mL) |
| Iba1 | Rabbit polyclonal anti-Iba1 (019-19741) | Wako* | 0.01M sodium citrate buffer, pH 6.0<br><br>Overnight at 60°C | 1:500 (1µg/mL) |
| CD3 | Rabbit polyclonal anti-human CD3 (A0452) | Dako | Proteinase K (Dako)<br><br>20 minutes at RT | 1:50 (8µg/mL) |
| Pax5 | Mouse monoclonal anti-human B-cell-specific activator protein, clone DAK-Pax5 (M7307) | Dako | 0.01M sodium citrate buffer, pH 6.0<br><br>20 minutes at 121°C§ | 1:50 (3.14µg/mL) |

**Table 2.3:** Summary of the primary antibodies used and antigen retrieval methods for immunohistochemical investigation of the cell populations present in feline ocular tissues with mycobacterial lesions. M = molar.

*Wako, Osaka, Japan

§Antigen retrieval performed using the Antigen Retriever 2100 (Aptum Biologics Ltd, Southampton, UK)

## 2.3 Results

### 2.3.1 Study Population Characteristics

Twenty-six globes with a morphological diagnosis of mycobacteriosis were identified from archives and assessed for eligibility. Two globes were excluded due to lack of preservation of globe morphology and loss of definition of ocular structures, resulting in a final study population of 24 globes from 24 cats. Summarised details are available in Table 2.4. The median age of cats with ocular mycobacteriosis was 6 years (range: 1 year – 12 years, 6 months). There were slightly more male cats included in this study (13 male, 11 female); the neuter status was known for 19 individuals (MN, n = 11; FN, n = 8). The most common breed was domestic short/medium/longhair (DxH) (n = 18); there were three British Shorthair (BSH), one Bengal and one Burmese, respectively, and the breed was not recorded for one animal. Ten of the cases presented in this study were described by Stavinohova *et al.*, 2019, and are highlighted in Table 2.4.

Results of specialist culture, PCR or IGRA were available for 22 cats; a diagnosis of TB *i.e.,* infection with a member of the MTBC was made in 20/22 cats (91%) (cases 1-20). Mycobacterial culture was performed in three cases and was positive for *M. bovis* in one cat (case 1), and *M. microti* infection in two cats (cases 2 and 3). These three cats also underwent testing by IGRA, the results correlating with the culture-confirmed diagnosis (Rhodes *et al.*, 2011). Six cats were diagnosed with *M. bovis* infection by PCR (Genotype Mycobacterium and GenotypeMTBC kits, Hain Lifescience GmbH, Nehren, Germany) (Kirscher *et al.*, 1993, Richter *et al.*, 2003) (cases 4-9), while seven further cats had a positive PCR result confirming MTBC infection, but further testing to identify the mycobacterial species was not performed either due to insufficient DNA, financial restrictions or lack of test availability (cases 10-16). A concurrent IGRA was performed in three of these cats which indicated infection with *M. bovis* in one cat (case 10), and MTBC infection in the other two cats (cases 11-12). A further four cats were diagnosed by IGRA, with *M. bovis* infection suggested in three cats (cases 17-19) and MTBC infection in the remaining individual (case 20); this cat had consumed the commercial raw food associated with an outbreak of *M. bovis* TB (O'Halloran *et al.*, 2020). A diagnosis of *M. lepraemurium* was made in 2/22 cats (9%) by PCR and sequencing of the 16S rRNA product (cases 21 and 22). Culture, PCR or IGRA was not performed for the remaining two cats (cases 23 and 24), therefore a species level diagnosis of mycobacterial infection was not attained for these two cases. Results of FeLV antigen and FIV antibody testing were available for four cats, and all were negative (cases 1, 10, 17 and 19).

| Case | Age | Gender | Breed | Culture | PCR | IGRA | FeLV antigen | FIV antibody | Stavinohova *et al.*, 2019 Case Number |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 6y | MN | Bengal | *M. bovis* | Negative | *M. bovis* | Negative | Negative | 10 |
| 2 | 8y | FN | DSH | *M. microti* | NP | MTBC | NP | NP | 6 |
| 3 | 11y | MN | DSH | *M. microti* | NP | MTBC | NP | NP | - |
| 4 | 10y | MN | BSH | NP | *M. bovis* | NP | NA | NA | 20 |
| 5 | 4y | F- | DxH | NP | *M. bovis* | NP | NA | NA | - |
| 6 | 8y | MN | DSH | NP | *M. bovis* | NP | NA | NA | 21 |
| 7 | 7y | F- | DxH | NP | *M. bovis* | NP | NA | NA | - |
| 8 | 1y | M- | NA | NP | *M. bovis* | NP | NA | NA | - |
| 9 | 5y 4m | FN | DSH | NP | *M. bovis* | NP | NA | NA | - |
| 10 | 10y 5m | MN | DSH | NP | MTBC | *M. bovis* | Negative | Negative | - |
| 11 | 3y | FN | DSH | NP | MTBC | MTBC | NP | NP | - |
| 12 | 7y | MN | DSH | NP | MTBC | MTBC | NP | NP | - |
| 13 | 4y | MN | DSH | NP | MTBC | NP | NA | NA | 14 |
| 14 | 2y | MN | DSH | NP | MTBC | NP | NA | NA | 17 |
| 15 | 6y | MN | BSH | NP | MTBC | NP | NA | NA | 18 |
| 16 | 11y | M- | DSH | NP | MTBC | NP | NA | NA | - |
| 17 | 7y 2m | MN | Burmese | NP | NP | *M. bovis* | Negative | Negative | 3 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 18 | 1y 9m | MN | DSH | Negative | Negative | *M. bovis* | NP | NP | 2 |
| 19 | 2y 1m | FN | DSH | NP | NP | *M. bovis* | Negative | Negative | - |
| 20 | 2y | FN | BSH | NP | NP | MTBC | NP | NP | - |
| 21 | 12y 6m | FN | DLH | NP | *M. lepraemurium* | NP | NA | NA | - |
| 22 | 10y | F- | DxH | NP | *M. lepraemurium* | NP | NA | NA | - |
| 23 | 3y | FN | DLH | NP | NP | NP | NP | NP | - |
| 24 | 1y 6m | FN | DSH | NP | NP | NP | NP | NP | 22 |

**Table 2.4:** Summary of case details for cats included in this study, including signalment data, results of diagnostic testing for mycobacterial infection, and results of testing for FeLV and FIV. y = years. m = months. M- = male, unknown neuter status. F- = female, unknown neuter status. NP = not performed. NA = not available.

## 2.3.2 Lesion Distribution and Inflammation Score

Histopathological examination of H&E-stained sections identified changes consistent with mycobacterial infection *i.e.,* granulomatous to pyogranulomatous inflammation with epithelioid macrophages, with or without necrosis, or infiltration with non-specific inflammatory cells present across a range of ocular tissues, with varying degrees of involvement and severity within and between individuals (Table 2.5). Inflammatory cells were identified most often within the choroid, retina, ciliary body and sclera, 20/24 cats (83%). There was wide variation in inflammation scores across tissues (Figure 2.1); where inflammation was present the highest median inflammation score was recorded for choroidal lesions (median score = 5). All cats with choroidal lesions showed concurrent retinitis, although the degree of inflammation was less pronounced (median score = 3). Posterior or panuveitis with retinitis was the most common histological finding, recorded in 20/24 cats (83%) (Table 2.6; cases 1, 2, 4-14, 16-20, 23 and 24). An appreciable inflammatory cell component was present within the vitreous cavity in three of these cats (cases 8, 17 and 18), resulting in an endophthalmitis. Anterior uveitis without choroidal involvement was identified in one cat (case 15). Histopathological evidence of optic neuritis was seen in 11/20 cats (55%) (cases 1, 6-9, 12-14, 20, 23 and 24); the optic nerve was not present for evaluation in four cats (cases 4, 5, 21 and 22). Anterior mass lesions affecting the cornea, bulbar or palpebral conjunctiva and/or sclera were recorded in 5/24 cats (21%) (cases 2, 3, 16, 21 and 22); inflammation restricted to these tissues with no intraocular involvement was present in three of the five cats. In the two cats with *M. lepraemurium* infection, lesions were restricted to the cornea and sclera (case 21), and in one cat there was also infiltration of the bulbar conjunctiva with inflammatory cells (case 22). A conjunctival mass was the only histopathological finding in one cat diagnosed with *M. microti* (case 3), whereas posterior or panuveitis was recorded in the remaining cats that had a diagnosis of MTBC infection.

## 2.3.3 Acid-Fast Bacilli

Ziehl-Neelsen staining demonstrated the presence of AFB morphologically consistent with mycobacteria in 20/24 globes (83%) (Table 2.5, cases 1-14, 16-21), although in two cats only one AFB was identified across all affected tissues (cases 19 and 20). Acid-fast bacilli were identified in 16/20 (80%) of choroidal lesions (cases 1, 2, 4-14, 17, 19 and 20); the median BI grade for choroidal lesions was 3, compared to 0 for the other tissues examined, although there was a wide range of BI grades observed across tissues (Figure 2.2). Of the five cats with a mass lesion present in the anterior segment of the globe (cornea, bulbar or palpebral conjunctiva and anterior sclera), AFB were detected in four of these (cases 2, 3, 16 and 21), with at least one of the affected tissues per case scoring as high BI (grade 3 or above). Acid-fast bacilli were only observed in regions of (pyo)granulomatous inflammation, and extracellular AFB were frequently identified in regions of necrosis, sometimes in very large numbers.

| Case | Cornea | | | Conjunctiva | | | Sclera | | | Iris | | | Ciliary Body | | | Choroid | | | Retina | | | Optic Nerve | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Score | Type | BI | Score | Type | BI | Score | Type | BI | Score | Type | BI | Score | Type | BI | Score | Type | BI | Score | Type | BI | Score | Type | BI |
| 1 | - | - | - | - | - | - | 3 | PG | 4 | - | - | - | 1 | LP | 0 | 5 | PG | 4 | 2 | PG | 0 | 3 | MX | 0 |
| 2 | 5 | PG | 3 | 3 | MX | 2 | 3 | MX | 4 | 2 | LP | 0 | 4 | MX | 3 | 4 | MX | 4 | 2 | PG | 0 | - | - | - |
| 3 | - | - | - | 5 | PG | 4 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 4 | - | - | - | 2 | MX | 0 | 5 | PG | 2 | 2 | LP | 0 | 3 | MX | 0 | 5 | MX | 3 | 2 | MX | 3 | NA | - | - |
| 5 | - | - | - | - | - | - | 1 | MX | 0 | 2 | LP | 0 | 2 | MX | 0 | 4 | MX | 3 | 4 | MX | 2 | NA | - | - |
| 6 | - | - | - | - | - | - | 1 | MX | 0 | 2 | MX | 0 | 3 | PG | 4 | 3 | PG | 4 | 2 | MX | 4 | 3 | PG | 3 |
| 7 | - | - | - | - | - | - | 3 | PG | 0 | 2 | LP | 0 | 3 | MX | 0 | 5 | PG | 5 | 3 | PG | 4 | 3 | PG | 0 |
| 8 | - | - | - | - | - | - | 2 | PG | 2 | - | - | - | 1 | PG | 0 | 5 | PG | 5 | 3 | PG | 5 | 5 | PG | 5 |
| 9 | 2 | PG | 0 | 5 | PG | 5 | 5 | PG | 4 | 3 | MX | 3 | 5 | PG | 4 | 5 | PG | 4 | 3 | PG | 3 | 2 | LP | 0 |
| 10 | - | - | - | 1 | LP | 0 | 1 | MX | 0 | 1 | LP | 0 | 2 | LP | 0 | 5 | MX | 4 | 3 | MX | 3 | - | - | - |
| 11 | - | - | - | - | - | - | - | - | - | 2 | LP | 0 | 3 | PG | 3 | 4 | MX | 3 | 4 | MX | 0 | - | - | - |
| 12 | - | - | - | 2 | MX | 0 | 5 | PG | 0 | 1 | LP | 0 | 5 | PG | 1 | 5 | MX | 1 | 2 | LP | 0 | 5 | PG | 0 |
| 13 | - | - | - | 1 | MX | 0 | 1 | MX | 0 | 1 | LP | 0 | 1 | LP | 0 | 5 | PG | 3 | 4 | MX | 0 | 2 | PG | 0 |
| 14 | - | - | - | - | - | - | 2 | MX | 0 | 1 | LP | 0 | 2 | MX | 0 | 3 | MX | 2 | 4 | PG | 0 | 2 | MX | 0 |
| 15 | - | - | - | - | - | - | - | - | - | 3 | PG | 0 | 2 | LP | 0 | - | - | - | - | - | - | - | - | - |
| 16 | 4 | PG | 0 | 4 | PG | 0 | 5 | PG | 3 | 2 | LP | 0 | 3 | PG | 0 | 3 | PG | 0 | 2 | LP | 0 | - | - | - |

| | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 17 | - | - | - | - | - | - | 2 | MX | 0 | 5 | PG | 2 | 5 | PG | 4 | 5 | MX | 2 | 4 | PG | 3 | - | - | - |
| 18 | 1 | LP | 0 | - | - | - | 2 | MX | 0 | 2 | MX | 0 | 4 | MX | 0 | 3 | PG | 0 | 3 | MX | 2 | - | - | - |
| 19 | - | - | - | - | - | - | 3 | PG | 0 | - | - | - | 2 | PG | 0 | 5 | PG | 1 | 3 | LP | 0 | - | - | - |
| 20 | - | - | - | - | - | - | 2 | MX | 0 | - | - | - | 1 | LP | 0 | 5 | MX | 1 | 3 | LP | 0 | 2 | MX | 0 |
| 21 | 3 | PG | 5 | - | - | - | 2 | PG | 5 | - | - | - | - | - | - | - | - | - | - | - | - | NA | - | - |
| 22 | 2 | PG | 0 | 2 | PG | 0 | 4 | PG | 0 | - | - | - | - | - | - | - | - | - | - | - | - | NA | - | - |
| 23 | - | - | - | 2 | MX | 0 | - | - | - | 2 | LP | 0 | 2 | MX | 0 | 3 | MX | 0 | 3 | MX | 0 | 2 | LP | 0 |
| 24 | - | - | - | - | - | - | 2 | MX | 0 | - | - | - | - | - | - | 5 | MX | 0 | 3 | MX | 0 | 2 | MX | 0 |

**Table 2.5:** Summary of inflammation score, inflammation type and BI grade per tissue examined per case. PG = pyogranulomatous inflammation. LP = lymphoplasmacytic inflammation. MX = mixed (pyo)granulomatous and lymphoplasmacytic inflammation.

| | Number of cases | Percentage (%) | Case |
|---|---|---|---|
| Posterior or panuveitis with retinitis | 20 | 83.3 | 1, 2, 4-14, 16-20, 23-24 |
| Optic neuritis | 11 | 55.0* | 1, 6-9, 12-14, 20, 23, 24 |
| Corneal, conjunctival and/or scleral mass | 5 | 20.8 | 2, 3, 16, 21, 22 |
| Orbital to periorbital cellulitis or abscess | 3 | 12.5 | 4, 9, 12 |
| Anterior uveitis | 1 | 4.2 | 15 |

**Table 2.6:** Summary of the main histological findings in cases of feline ocular mycobacteriosis. *Optic nerve not present in H&E-stained sections in four cases for evaluation.

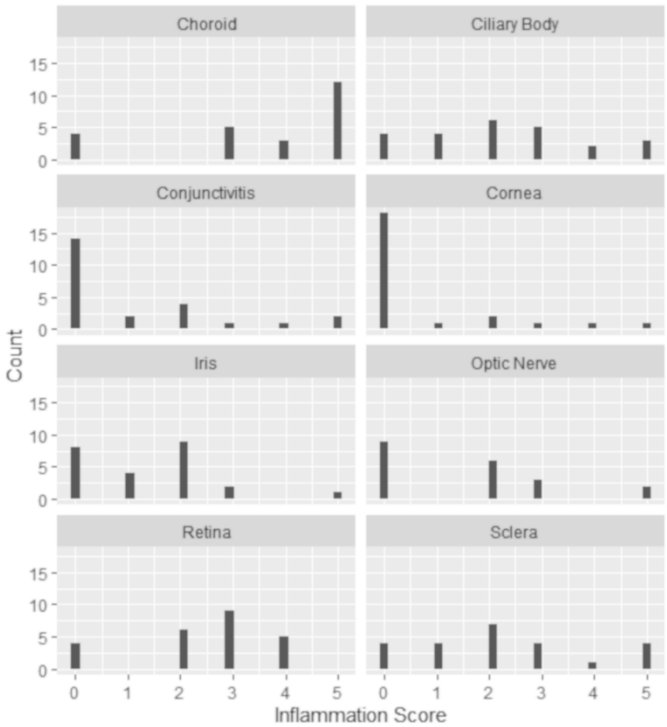


**Figure 2.1:** A histogram to show the inflammation score and location of ocular lesions identified in cases of feline ocular mycobacteriosis on H&E-stained tissue sections.

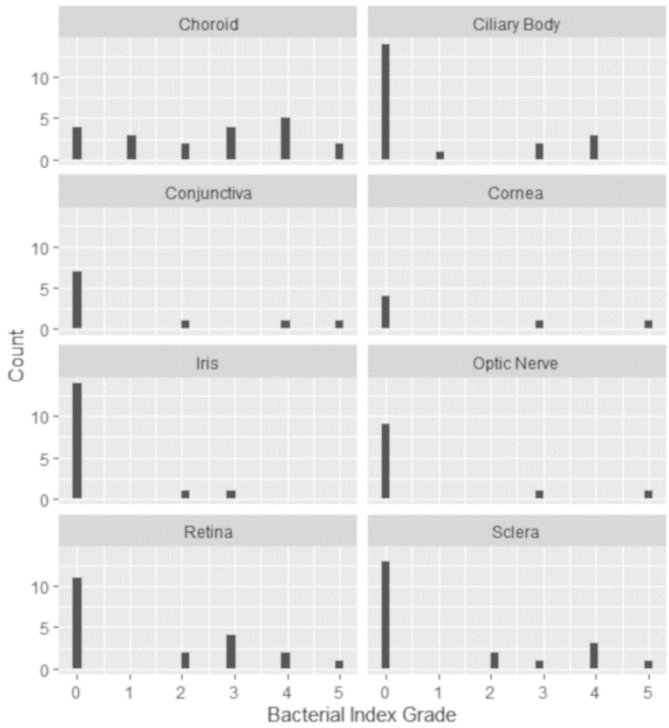


**Figure 2.2:** A histogram to show the BI grade for inflammatory lesions split by tissue type in cases of feline ocular mycobacteriosis, assessed on ZN-stained tissue sections.

## 2.3.4 Choroid and Retina

Choroidal lesions were recorded in 20/24 cases (83%) (Figure 2.3), and of these AFB were identified in 16/20 cases (80%) (Figure 2.4). The median inflammation score for choroidal lesions was higher than that for lesions in any other ocular tissue (median score = 5). The inflammatory population was ascribed as mixed in 11 cats (cases 2, 4, 5, 10-12, 14, 17, 20, 23 and 24), whereas the remaining nine cats showed predominantly (pyo)granulomatous inflammation (cases 1, 6-9, 13, 16, 18 and 19). Granulomatous/pyogranulomatous inflammation was present across all layers of the choroid, but this often became restricted to the inner choroid in regions approaching the pars plana. This is in comparison to the lymphoplasmacytic infiltrate, which was mostly restricted to perivascular aggregates around the large and medium vessels in the outer choroid, although five cats did show segmental lymphoplasmacytic infiltration within regions of (pyo)granulomatous inflammation (cases 1, 2, 11, 20 and 24). 'Organised' (pyo)granulomas were present in nine cats (cases 4, 5, 7, 9, 10, 16, 19, 20 and 24), some of which were surrounded by an outer layer of concentric spindle-shaped cells, but encapsulating fibrosis was often minimal; there was one cat with 'atypical' granulomas and abundant collagen deposition. There was no clear formation of (pyo)granulomas in 10 cats (cases 1, 2, 6, 8, 11, 13, 14, 17, 18 and 23); rather, the infiltrate formed a diffuse sheet of inflammatory cells, from herein termed 'unstructured'. Multifocal zones of necrosis within regions of (pyo)granulomatous inflammation ('organised' or 'atypical' (pyo)granulomas and unstructured sheets of inflammatory cells) were present in 17/20 (85%) cats (cases 1, 4-13, 16-20 and 24); concurrent degenerative change *e.g.,* vacuolation,

and necrosis of the *tapetum lucidum* was also identified. Diffuse or segmental choroidal fibrosis was identified in 12/20 (60%) cats (cases 1, 4-6, 9, 10, 14, 16, 17, 19, 20 and 24), especially of the choriocapillaris layer while sparing the larger choroidal vessels (Figure 2.5). On IHC, the most abundant cell type were Iba1-positive macrophages and epithelioid macrophages (Figure 2.6). The intensity of staining was greater for macrophages at the periphery of (pyo)granulomas, where present, with less intense staining in macrophages at the centre of the (pyo)granuloma. Calprotectin-positive monocytes and granulocytes were the second most abundant cell population present, often scattered throughout the (pyo)granulomatous inflammation and surrounding regions of necrosis. T-cells were the next most common population, often forming peripheral cuffs particularly around 'organised' (pyo)granulomas (Figure 2.7), or diffusely scattered throughout 'unstructured' (pyo)granulomatous lesions. In contrast, B-cells were rarely associated with (pyo)granulomas or 'unstructured' (pyo)granulomatous inflammation; these were the predominant cell type in regions of segmental lymphoplasmacytic inflammation (Figure 2.8), and rarely formed small clusters within choroidal (pyo)granulomatous lesions.

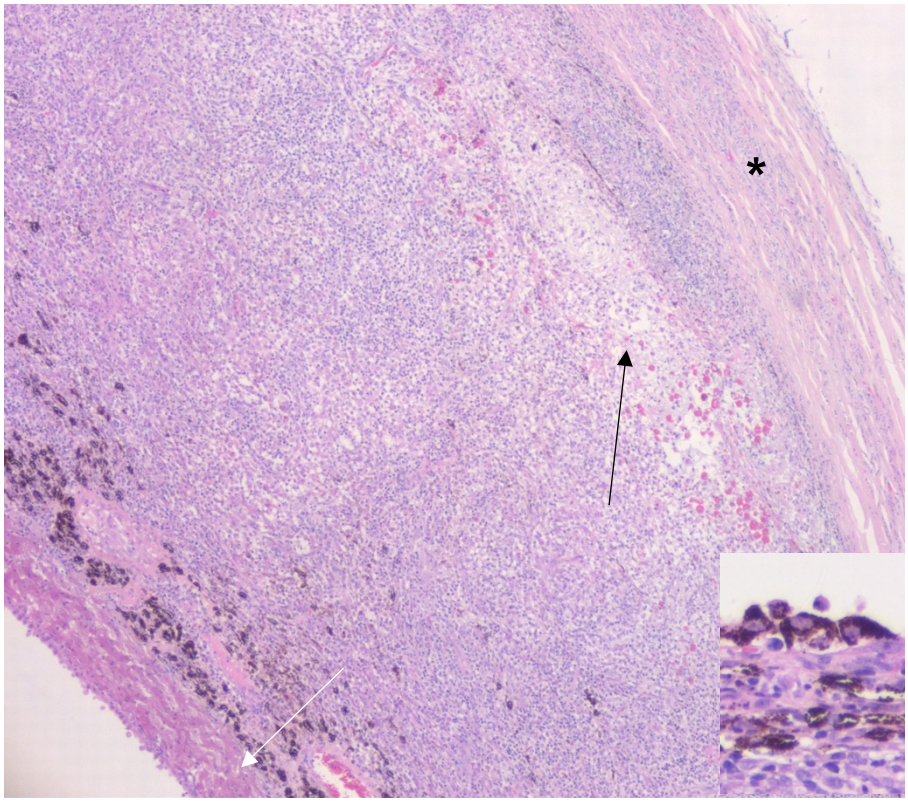


**Figure 2.3:** Case 7, pyogranulomatous choroiditis in a case of *M. bovis* infection, with necrosis and oedema (black arrow), infiltration of the sclera with inflammatory cells (asterisk), necrosis of the *tapetum lucidum* (white arrow) and hypertrophy of the retinal pigment epithelium (RPE) indicative of retinal detachment. x40 magnification (inset: x400 magnification).

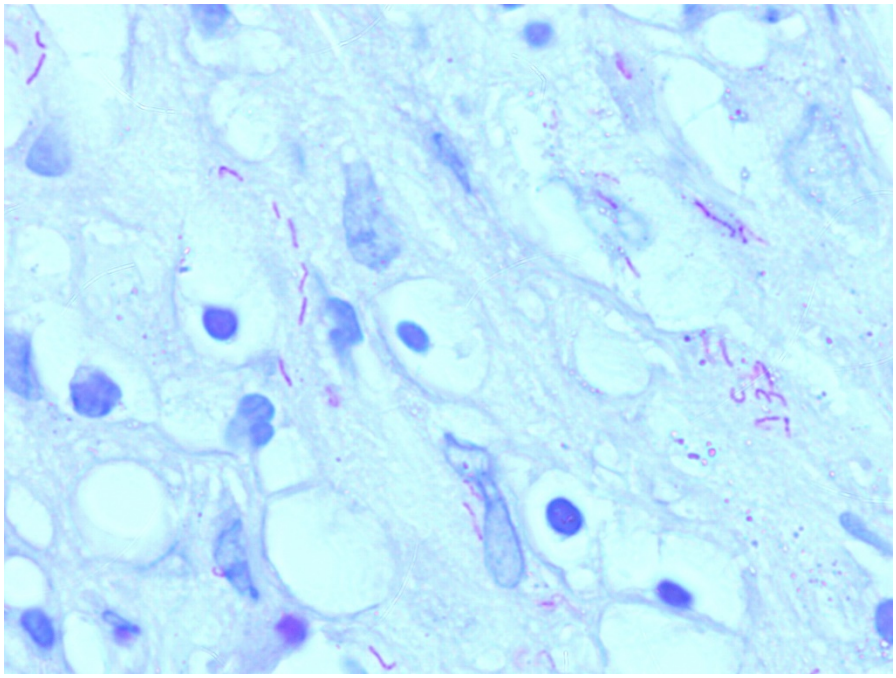

**Figure 2.4:** Case 2, pyogranulomaotous choroiditis with AFB on ZN-staining, some of which display the S-shaped morphology suggestive of *M. microti*. x1000 magnification.

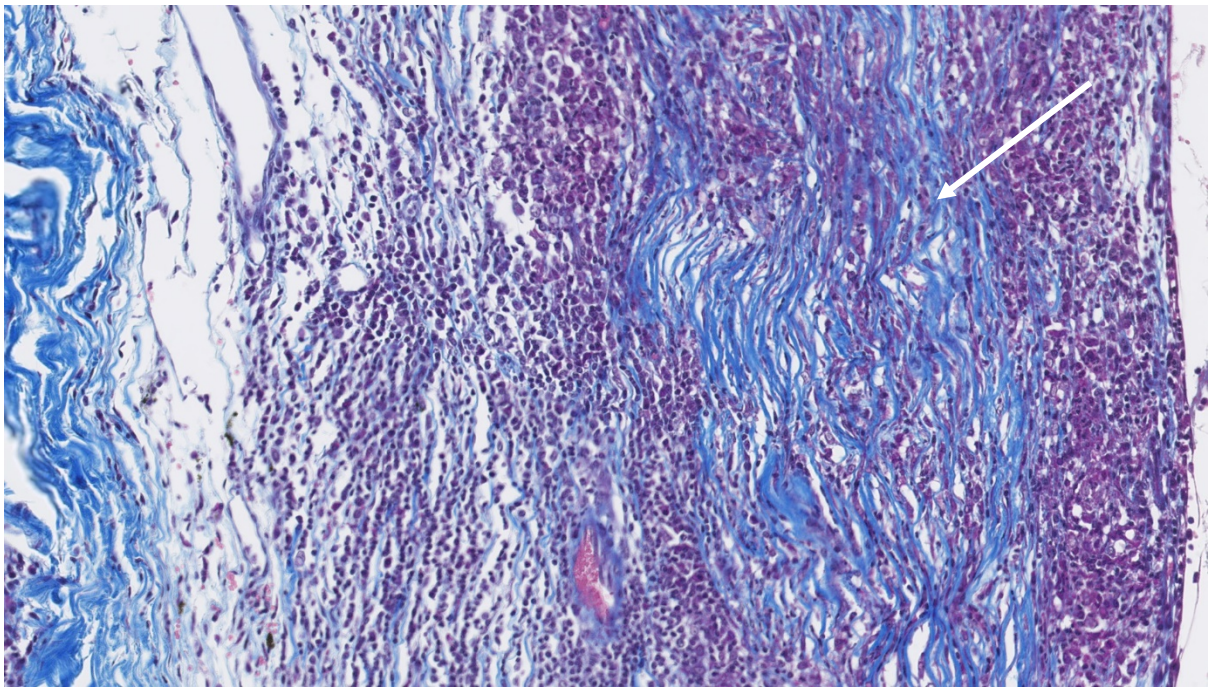

**Figure 2.5:** Case 1, expansion of the choroid with mixed inflammation and concurrent choroidal fibrosis (white arrow) demonstrated on MT staining in a case of *M. bovis* infection. x200 magnification.

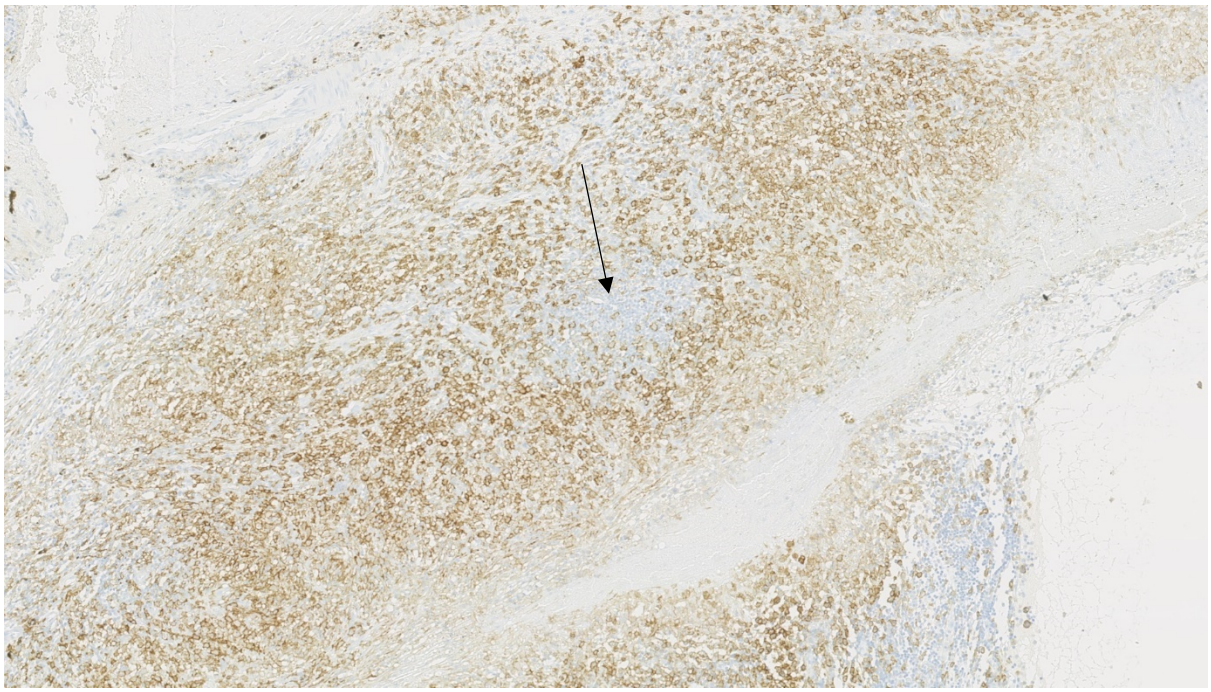

**Figure 2.6:** Case 13, abundant positive staining for Iba1 on epithelioid macrophages, and lack of positive staining on a cluster of lymphocytes (black arrow). This cat was diagnosed with an MTBC infection. x40 magnification.

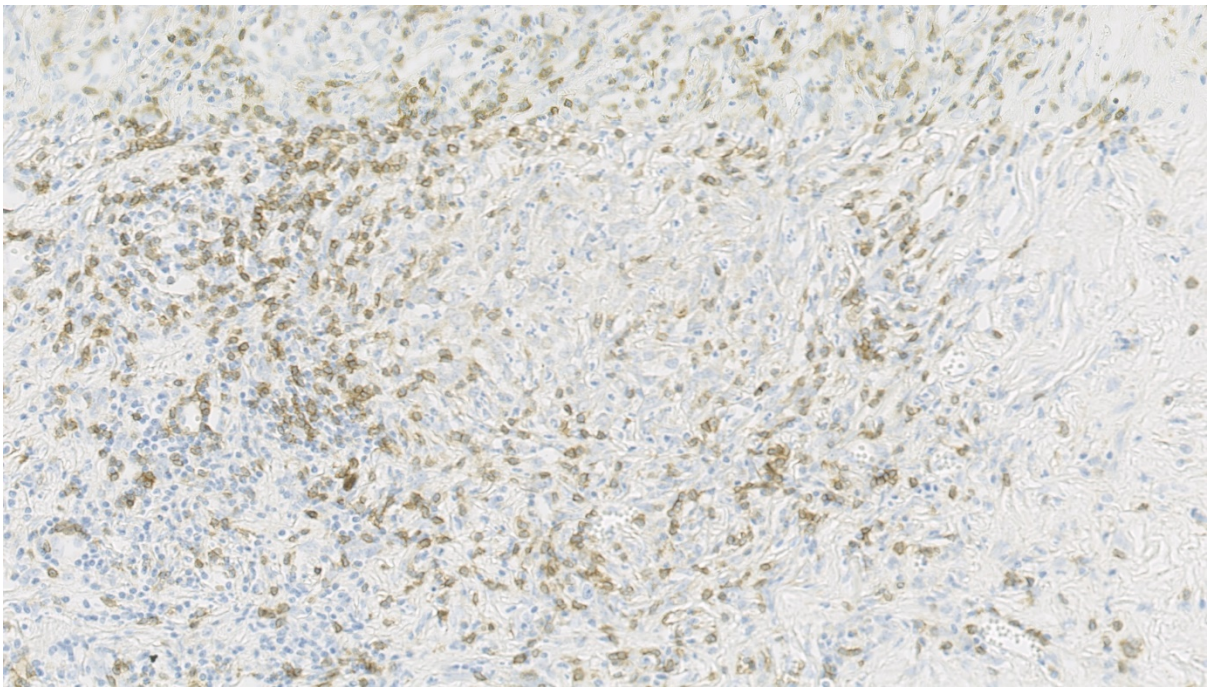

**Figure 2.7:** Case 4, positive membraneous staining with CD3 IHC for T-cells, forming a cuff around a granuloma in a case of *M. bovis* infection. x200 magnification.

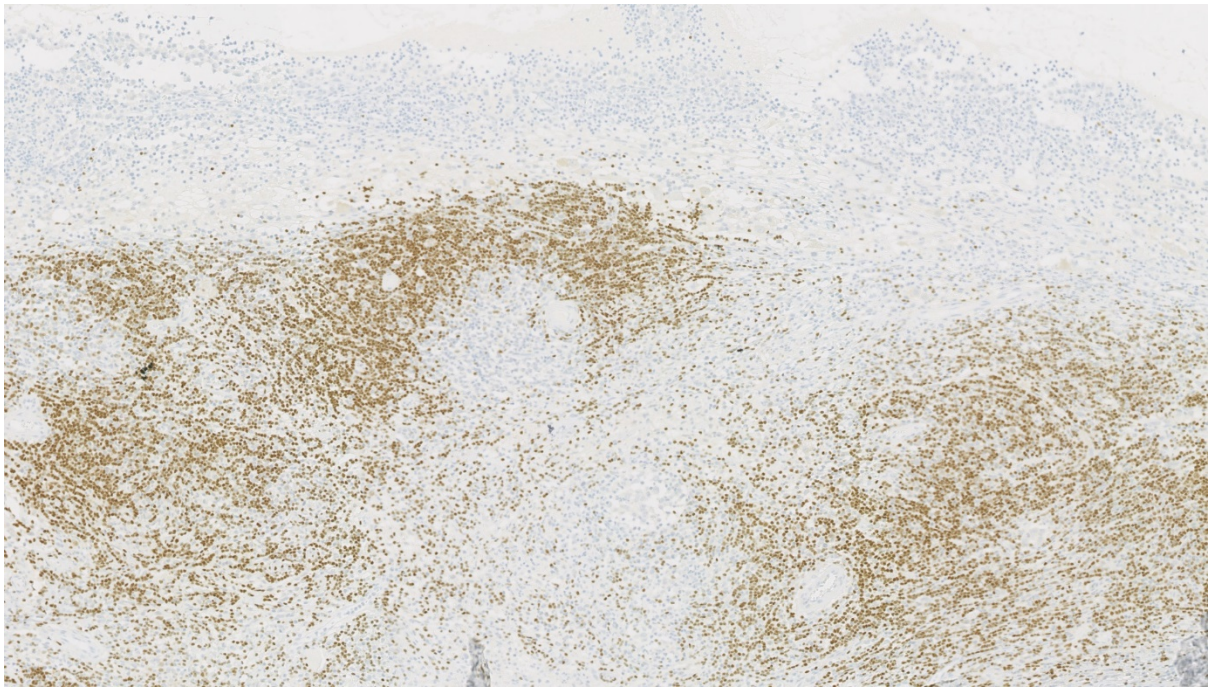

**Figure 2.8:** Case 17, large numbers of B-cells infiltrating the choroid in a cat infected with *M. bovis*. x40 magnification.

Retinal lesions were identified in the same 20/24 cats (83%) that had lesions within the choroid, although the median inflammation score was lower (median score = 3). (Pyo)granulomatous inflammation was the dominant finding in seven cats (cases 1, 2, 7-9, 14 and 17), with a mixed inflammatory population in nine cats (cases 4-6, 10, 11, 13, 23 and 24) (Figure 2.9). 'Organised' granulomas were identified in three cats (cases 5, 13 and 18); otherwise, the inflammatory cell infiltrate was 'unstructured'. The remaining four cats were dominated by lymphoplasmacytic inflammation (cases 12, 16, 19 and 20). Aggregates of perivascular lymphocytes were identified in 13 cats (cases 1, 4-7, 10, 13, 14, 16, 18, 19, 20 and 23); these cuffs comprised both T- and B-cells in variable numbers. Focal or complete retinal detachment, or evidence of *ante-mortem* retinal detachment *i.e.,* hypertrophy of the RPE and/or subretinal inflammatory exudate, was present in 16/20 cats (80%) with retinal lesions (cases 1, 5-8, 10-14, 16-20 and 23). Within the subretinal space a proteinaceous exudate, or proteinaceous debris, was identified in 15/16 cats (94%) (cases 1, 5-8, 10-14 and 16-20); a concurrent cellular component was identified in 14/15 cats (93%) (cases 1, 5-8, 10-14, 15 and 17-20), mostly consisting of Iba1- or calprotectin-positive cells, with fewer lymphocytes. Evidence of haemorrhage into the subretinal space *i.e.,* erythrocytes and erythrophages, was seen in 8/16 cats (50%) (cases 1, 8, 11, 13, 14, 18, 19 and 23). There was diffuse atrophy of the photoreceptor layer in all 20 cases; loss of the outer retinal layers was variable and, in some cases, this extended to full thickness retinal atrophy. Retinal necrosis was present in 15/20 cats (75%) and was typically diffuse or multifocal (cases 2, 4-11, 13, 14, 17-19 and 23). In cases of (pyo)granulomatous or mixed (pyo)granulomatous-lymphocytic retinitis Iba1-positive cells were the most common cell population on IHC; some positive cells formed

granulomas. There were positive cells on the inner limiting membrane of the retina, as well as scattered diffusely throughout multiple retinal layers (Figure 2.10); some of these likely represented reactive and quiescent resident microglia, in addition to infiltrating macrophages. Small numbers of Iba1-positive cells were also identified in cases of lymphocytic retinitis. Calprotectin-positive cells were also present throughout the retina, but numbers of positive cells decreased towards the periphery of the retina. T-cells were identified in all retinal lesions, often in association with areas of (pyo)granulomatous inflammation, but they were also diffusely scattered throughout the outer and inner retinal layers. As with choroidal lesions, B-cells were less likely to be found in association with areas of (pyo)granulomatous inflammation, however, in lymphocyte-rich regions of retinitis B-cells were identified, sometimes in huge numbers.

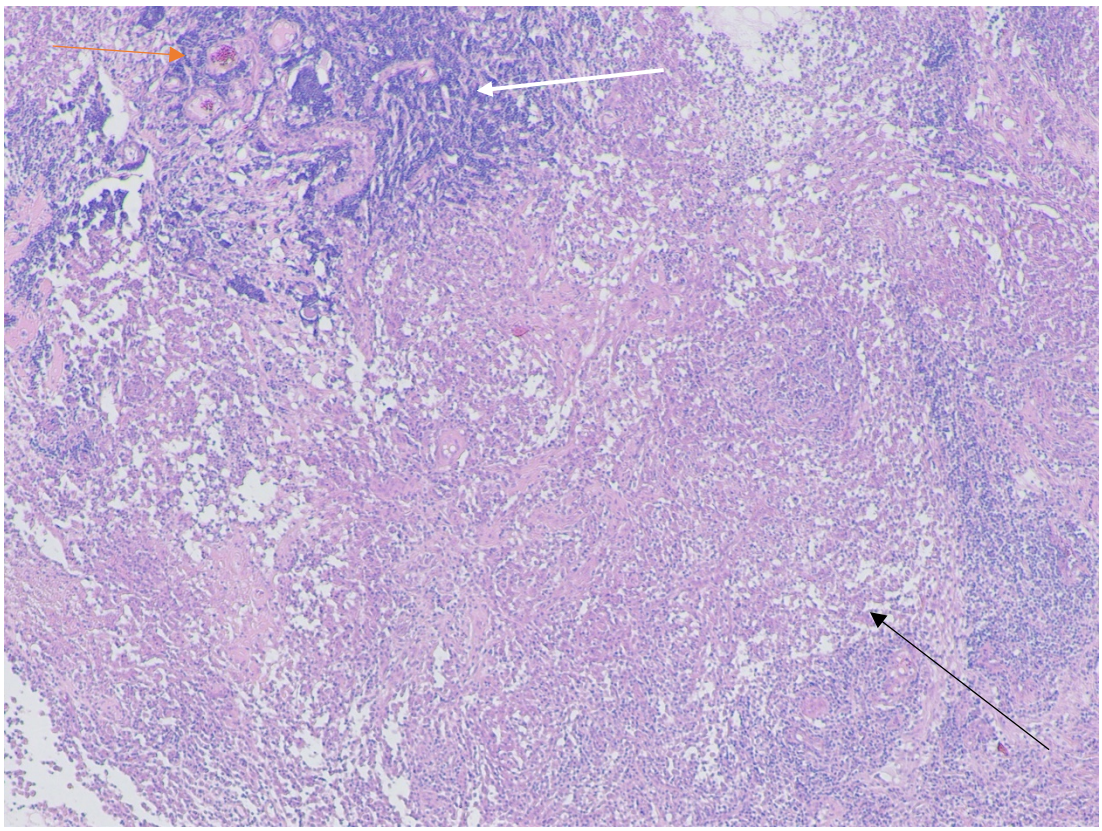

**Figure 2.9:** Case 13, mixed pyogranulomatous (black arrow) and lymphoplasmacytic (white arrow) retinitis due to MTBC infection. Note the perivascular accumulation of lymphocytes in the retina. x40 magnification.

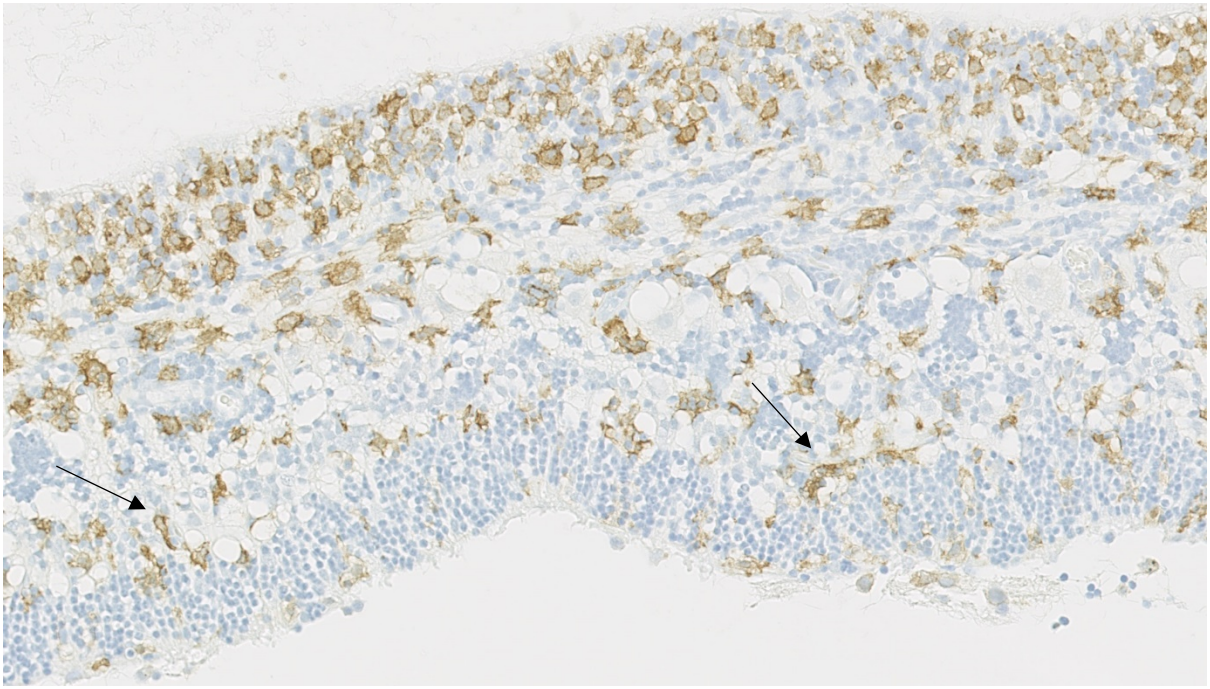

**Figure 2.10:** Case 18, Iba1-positive cells present within the inner layers of the retina, some of which have a more spindle-shaped morphology suggestive of resident microglia (black arrows). This cat was diagnosed with *M. bovis* infection. x200 magnification.

## 2.3.5 Anterior Uvea

Inflammatory cells were present in the iris of 16/24 cats (67%) (cases 2, 4-7, 9-18 and 23). Typically, iridal lesions were characterised by lymphoplasmacytic inflammation with an inflammation score of 1 to 2 (11/16 cats, 69%) (cases 2, 4, 5, 7, 10-14, 16 and 23); this was the only ocular structure where lymphoplasmacytic inflammation predominated. The remaining five cats showed (pyo)granulomatous inflammation, or a mixed infiltrate (cases 6, 9, 15, 17 and 18). Acid-fast bacilli were identified in two cats, both of which were characterised by pyogranulomatous inflammation; one scored as BI grade 2 (low) (case 17) and the other as BI grade 3 (high) (case 9). Lymphocytes and plasma cells often formed perivascular cuffs but were also identified throughout the iris stroma either forming loose aggregates of cells or were diffusely distributed; most cases showed greater numbers of B-cells (Figures 2.11 A-B). In cases with (pyo)granulomatous or mixed inflammation, there was evidence of granuloma formation dominated by Iba1-positive macrophages, variable numbers of calprotectin-positive cells and a peripheral layer of T-cells. Concentrically arranged spindle-shaped cells were sometimes present, but there was no evidence of a fibrous capsule on staining with MT. Iba1-positive cells were infrequently identified lining the anterior iris epithelium. Extensive necrosis was present in one cat (case 17, inflammation score 5), resulting in the loss of the posterior pigmented iris epithelium. Pre-iridal membranes were identified in 20/24 cats (83%) (cases 1, 2, 4-20 and 23), although in some cases these were very subtle, consisting of a focal, single-cell thickness cellular membrane on the anterior surface of the iris.

There was evidence of inflammation in the ciliary body in 20/24 cats (83%); all 16 cats with iridal inflammation demonstrated concurrent cyclitis (cases 2, 4-7, 9-18 and 23). Inflammation of the iris was not identified in the remaining four cats (cases 1, 8 and 20, inflammation score 1; case 19, inflammation score 2). Ten cats showed (pyo)granulomatous or mixed inflammation, with an inflammation score of 3 or greater. Granulomas were identified in seven cats ('organised' cases 4, 9, 11, 16 and 17, 'atypical' cases 2 and 12), whereas the remaining three cats showed diffuse 'unstructured' infiltration of the ciliary body (cases 6, 7 and 18). In two cats with an inflammation score of 5 there was extensive destruction and necrosis of the pars plicata (Figure 2.12) (cases 9 and 17).

In 14/24 cats (58%) there was extension of inflammatory cells from the base of the iris or the ciliary body into the trabecular meshwork (cases 2, 5-7, 9-12, 14-18 and 23), and in three cats there was collapse of the iridocorneal drainage angle (cases 6, 16 and 17). In 10 cats, this inflammatory cell population was predominated by Iba1- or calprotectin-positive cells, with variable numbers of T- and B-cells (cases 5, 6, 9, 11, 12, 14, 16-18, 23). Lymphoplasmacytic infiltration of the trabecular meshwork dominated in four cats, of which three showed greater numbers of T-cells (cases 2, 7 and 15), whereas B-cells dominated in the remaining cat (case 10). The optic disc was not present in the examined sections to evaluate for cupping, which would indicate glaucoma. However, there was clinical suspicion of glaucoma reported by the histopathologist in three cases (cases 6, 17 and 18).

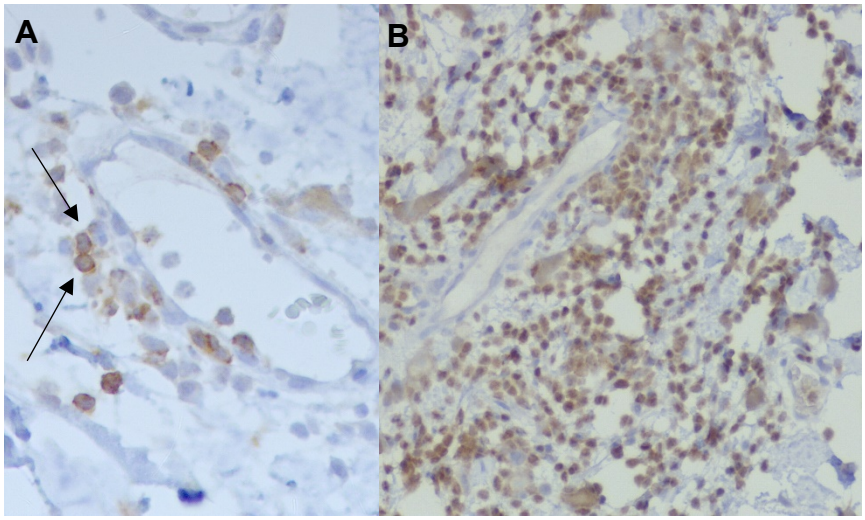


**Figure 2.11:** Case 5, mild lymphoplasmacytic iritis with scant T-cells displaying positive membranous staining (black arrows) (A) and more abundant B-cells (B) in a case of *M. bovis* infection. x400 magnification (A). x200 magnification (B).

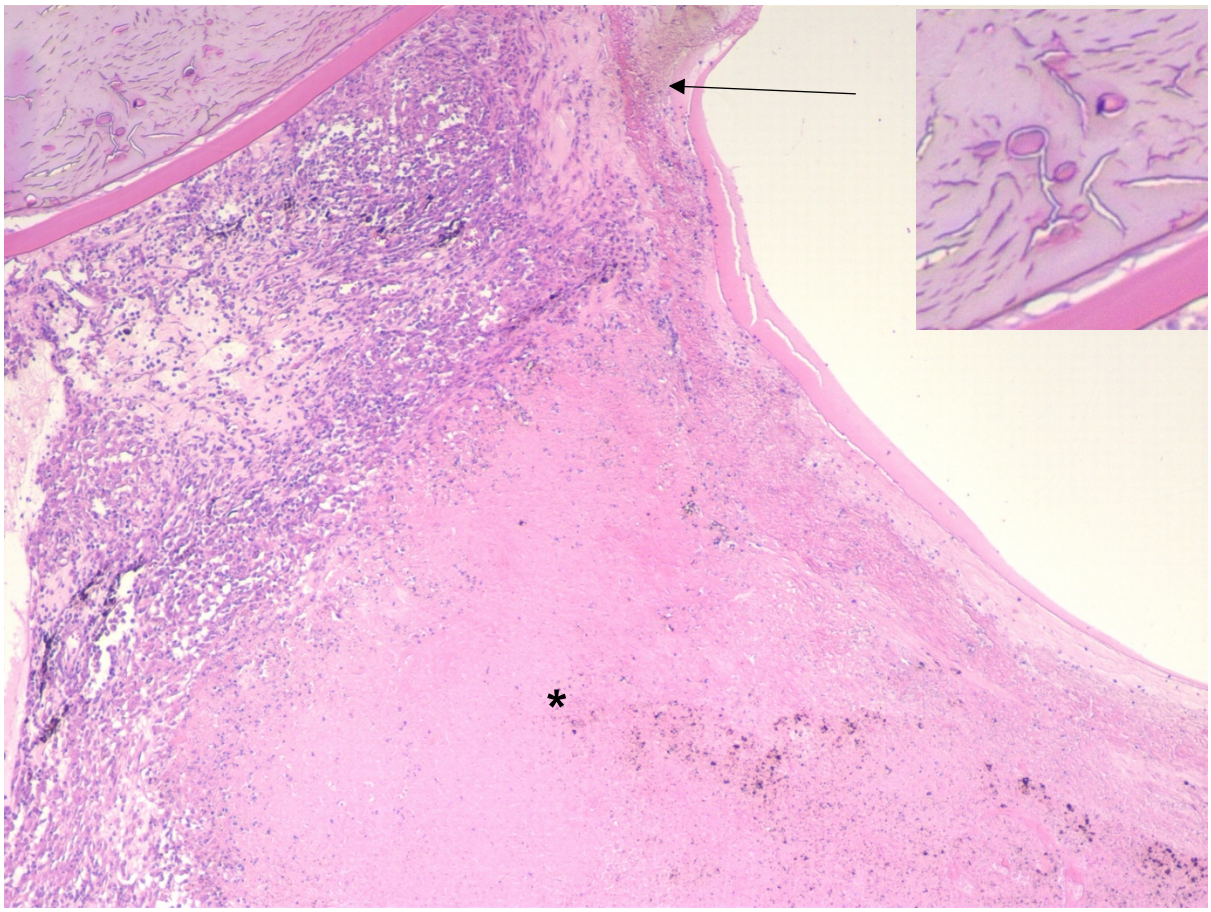

**Figure 2.12:** Case 9, pyogranulomatous cyclitis due to *M. bovis* infection with extensive necrosis (asterisk), and loss of the pars plicata. There is a cyclitic membrane (black arrow), and Morgagnian globules in the lens (see inset) indicating a cataract. x40 magnification.

## 2.3.6 Cornea, Sclera, and Conjunctiva

Inflammatory cells were present within the sclera in 20/24 cats (83%) (cases 1, 2, 4-10, 12-14, 16-22 and 24), with a median inflammation score of 2, although an inflammation score of 5 was recorded in four cats (cases 4, 9, 12 and 16). Perivascular mixed inflammation (inflammation score 1 to 2) was the sole scleral change identified in eight cats (cases 5, 6, 10, 13, 14, 17, 20 and 24), with a further two cats showing perivascular inflammation in conjunction with other inflammatory lesions within the sclera (cases 2 and 18). Extension of (pyo)granulomatous choroidal lesions into the posterior sclera was recorded in four cats (cases 1, 7, 8 and 19), a nodular (pyo)granulomatous anterior scleritis was present in four cats (cases 2, 16, 21 and 22), and (pyo)granulomatous scleritis as part of an orbital or periorbital cellulitis was identified in three cats (cases 4, 9 and 12). Granulomas or pyogranulomas were present in nine cats with (pyo)granulomatous inflammation (cases 2, 4, 9, 12, 16, 18, 19, 21 and 22), compared to three cats with an ‘unstructured’ inflammatory infiltrate (cases 1, 7 and 8). Where (pyo)granulomas were present, they were classified as ‘organised’ in four cats (cases 4, 9, 12 and 16); fibrous

encapsulation of (pyo)granulomas ranged from absent to thick, well-formed capsules. Scleral collagenolysis was identified in cats with an inflammation score of 4 or 5 (Figure 2.13). As with lesions in other tissues, Iba1-positive macrophages were the most common cell type on IHC, followed by calprotectin-positive cells (Figure 2.14), and with lesser numbers of T- and B-cells, and a similar pattern of distribution.

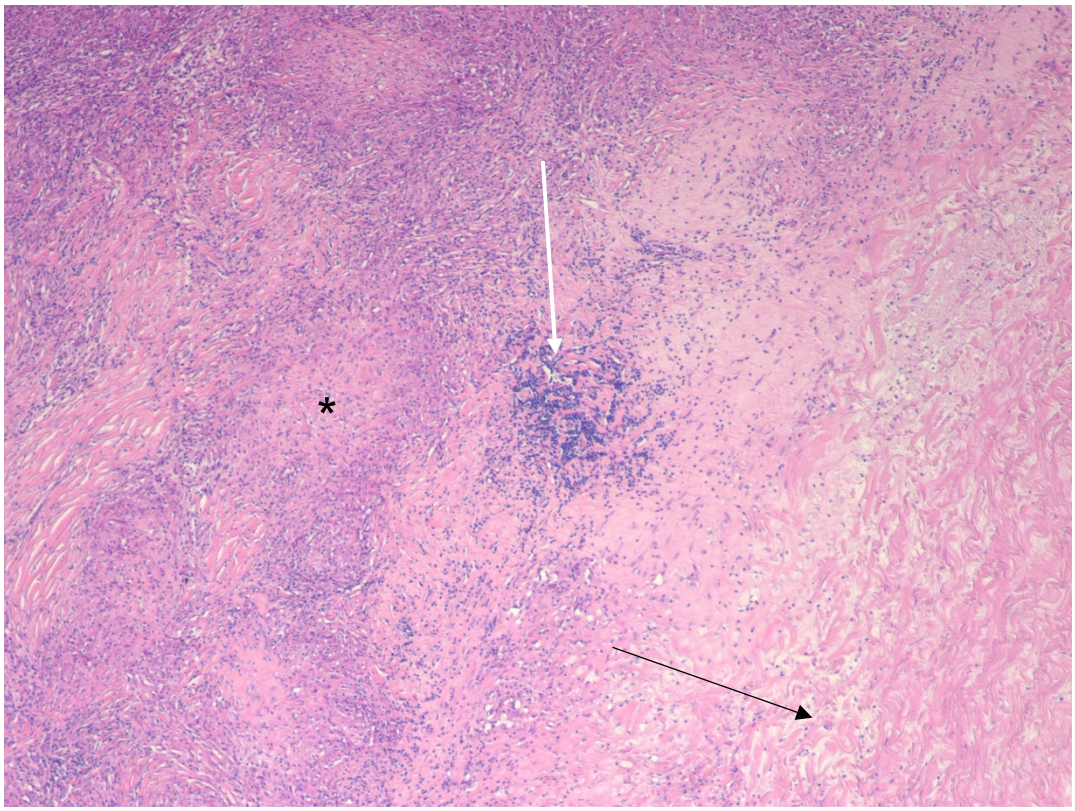


**Figure 2.13:** Case 4, pyogranulomatous scleritis and episcleritis due to *M. bovis* infection, with collagen degeneration (black arrow), small granulomas (asterisk) and clusters of lymphocytes (white arrow). x40 magnification.

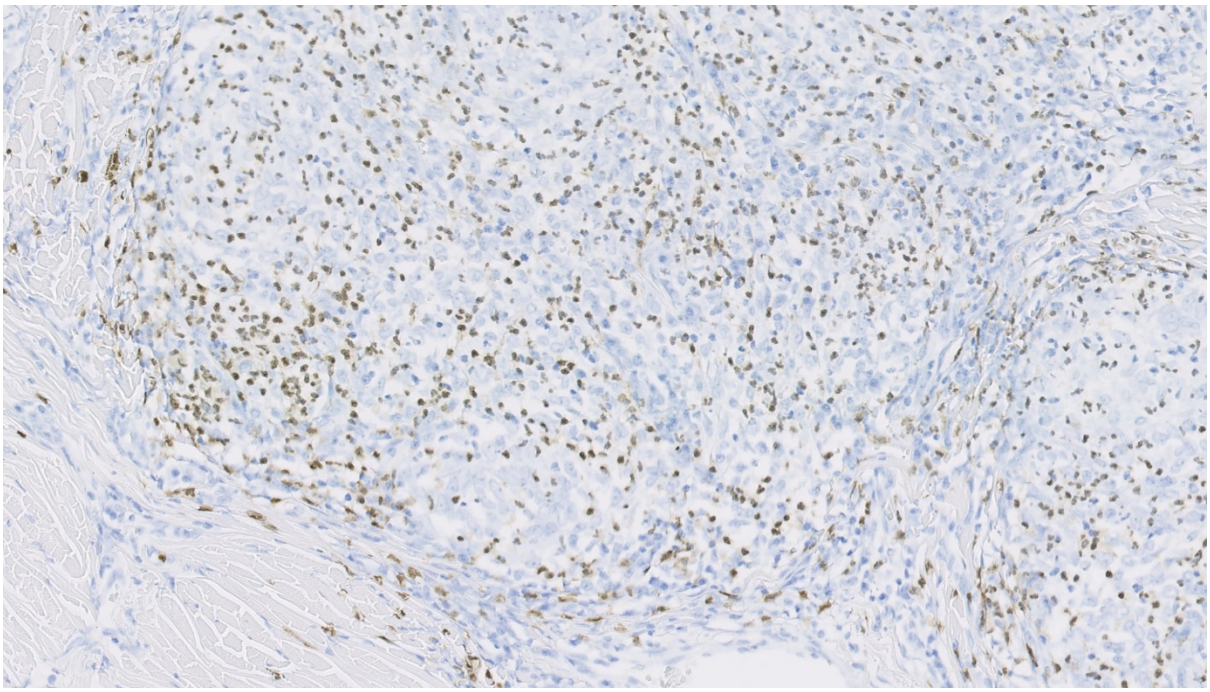

**Figure 2.14:** Case 22, pyogranulomatous scleritis in a case of *M. lepraemurium* infection, showing 'atypical' granulomas with scattered calprotectin-positive monocytes and granulocytes. x200 magnification.

Corneal lesions were identified in 6/24 cats (25%) (cases 2, 9, 16, 18, 21 and 22), with a median inflammation score of 2.5. Five of the lesions were (pyo)granulomatous, dominated by Iba1-positive epithelioid macrophages; four of these formed 'atypical' (pyo)granulomas with varying degrees of encapsulating fibrosis (cases 2, 9, 21 and 22) whereas case 16 showed 'organised' (pyo)granulomas. Corneal lesions were contiguous with a lesion that extended into the sclera, bulbar conjunctiva and episcleral tissues (cases 2, 16 and 22). In case 21 the corneal lesion was contained within the stroma (Figures 2.15 A-B). Corneal vascularisation was identified in all six cats.

Infiltration of the conjunctiva with inflammatory cells was recorded in 10/24 cats (42%) (cases 2-4, 9, 10, 12, 13, 16, 22 and 23), with a median inflammation score of 2. Two cats had an inflammation score of 5 (cases 3 and 9), both of which consisted of pyogranulomatous inflammation dominated by Iba1-positive macrophages and epithelioid macrophages and were scored with a high BI (grade 4 and 5, respectively). In one cat the inflammatory cell population was 'unstructured', with mixed regions of fibrosis, necrosis and oedema, as part of a periorbital cellulitis (case 9); case 3 consisted of a large confluent sheet of 'atypical' pyogranulomas diffusely expanding the nictitating conjunctiva. Extensive regions of fibrosis, with areas of necrosis, were also present (Figure 2.16). 'Atypical' granulomas with encapsulating fibrosis and bridging populations of T-cells were also identified in the second cat with *M. microti* infection (case 2); the conjunctiva was involved as part of a corneal-scleral-conjunctival mass. Another cat with a contiguous corneal-scleral-conjunctival mass consisted of 'organised'

pyogranulomas, dominated by Iba1-positive macrophages and calprotectin-positive cells, with variable degrees of fibrosis and some regions of necrosis (case 16). The remaining five cats showed diffuse infiltration with small numbers of calprotectin-positive cells, T-cells and fewer B-cells.

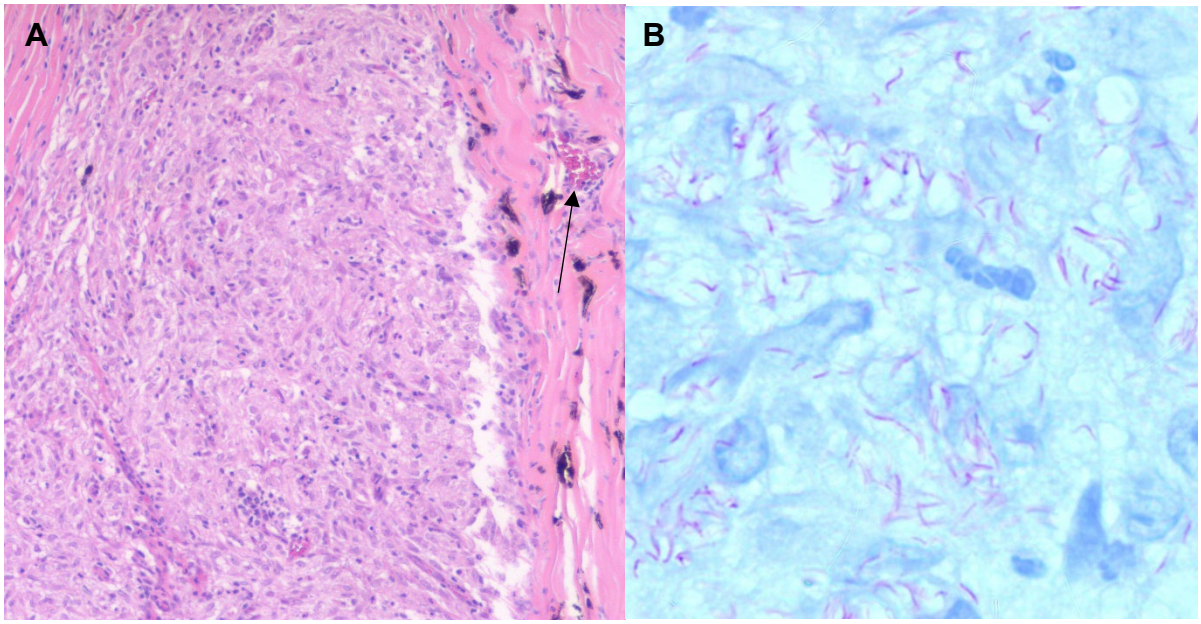


**Figure 2.15:** Case 21, pyogranulomatous keratitis due to *M. lepraemurium* infection expanding the corneal stroma with the formation of 'atypical' granulomas. Corneal vascularisation is also present (black arrow) (A). There are abundant ZN-positive organisms within the lesion (B). x200 magnification (A). x1000 magnification (B).

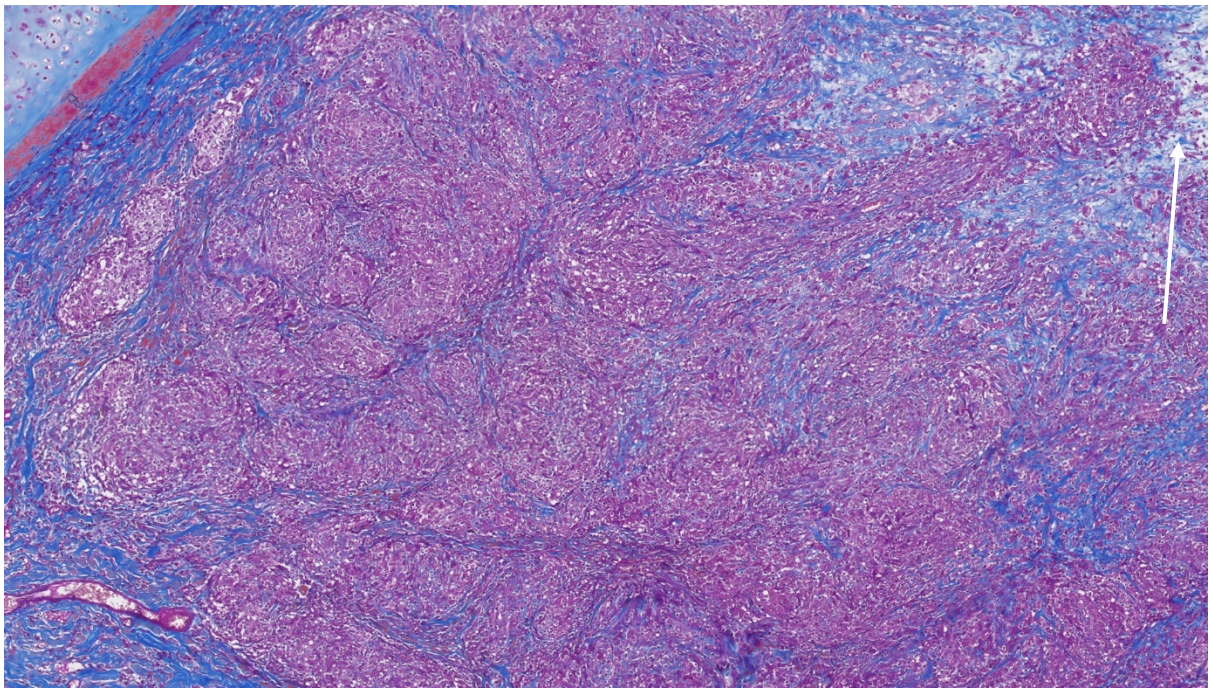

**Figure 2.16:** Case 3, pyogranulomatous conjunctivitis of the third eyelid resulting from *M. microti* infection, forming 'atypical' granulomas with a zone of collagen degeneration (white arrow). x40 magnification.

## 2.3.7 Optic Nerve

Inflammation of the optic nerve was present in 11/20 cats (55%) (cases 1, 6-9, 12-14, 20, 23 and 24), with a median inflammation score of 2; the optic nerve was not present for histopathological assessment in four cats (cases 4, 5, 21 and 22). Pyogranulomatous or mixed inflammation, dominated by Iba1-positive macrophages and epithelioid macrophages, was present in nine cats (cases 1, 6-8, 12-14, 20 and 24), compared to two cats (cases 9 and 23) where B-cells were the predominant cell type. In both cats, lymphocytes were found to accumulate around rather than directly infiltrate the optic nerve. Two cats had an inflammation score of 5. In one (case 12), small, mostly 'atypical' (pyo)granulomas were present, expanding the optic nerve; however, AFB were not detected in this tissue. Extensive necrosis and infiltration of the optic nerve with 'unstructured' pyogranulomatous inflammation with minimal fibrosis was identified in the second cat (case 8); AFB were numerous (BI grade 5), especially within regions of necrosis (Figures 2.17 A-B). In the remaining cats there was infiltration of the optic nerve head and pial trabeculae with pyogranulomatous inflammation and a lesser lymphocytic component.

## 2.3.8 Anterior Chamber, Lens, and Vitreous Chamber

A proteinaceous effusion, or proteinaceous debris such as fibrin clots was identified in the anterior chamber in nine cats (cases 6, 7, 9, 11, 13, 14, 17, 18 and 23); in these cases, plus an additional three (cases 2, 8 and 16), inflammatory cells were present, some of which appeared adherent to the corneal endothelium. These cells were mostly calprotectin-positive, with fewer T-cells and B-cells.

A cataract was identified in five cats (cases 2, 4, 9, 17 and 23), and posterior synechiae were present in three cats (cases 6, 17 and 18). Rupture of the posterior lens capsule with neutrophilic phakitis and intra-lenticular AFB was identified in one cat (case 17).

Predominantly calprotectin-positive inflammatory cells with fewer macrophages and lymphocytes were present in appreciable numbers within the vitreous in three cats (Figure 2.18) (cases 8, 17 and 18). Free AFB were present within the vitreous of the single case with posterior lens capsule rupture and phakitis (case 17).

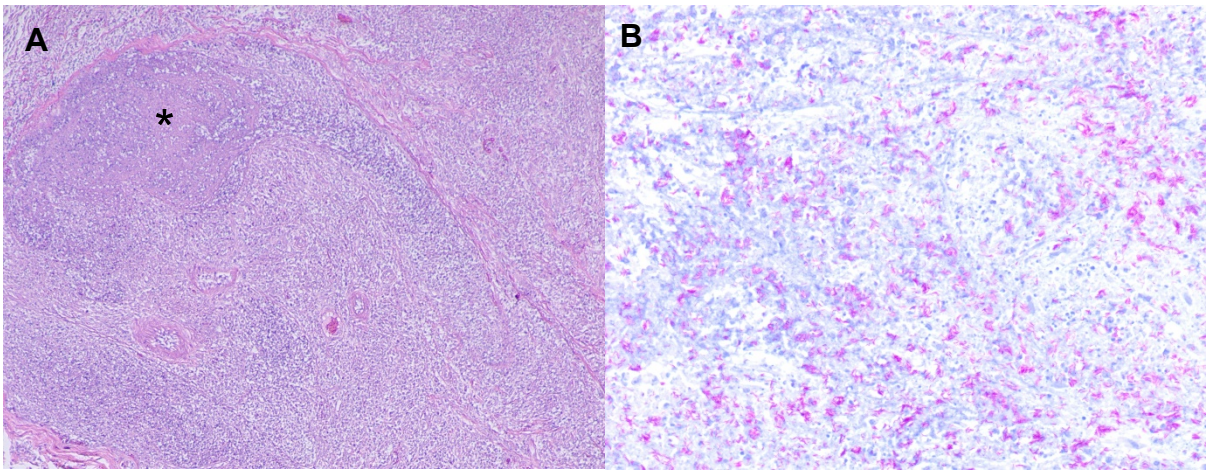


**Figure 2.17:** Case 8, pyogranulomatous optic neuritis due to infection with *M. bovis*. The optic nerve is expanded by the inflammatory cell infiltrate, and there is also necrosis (asterisk) (A) and huge numbers of AFB, BI grade 5, particularly within these necrotic regions (B). x40 magnification (A). x200 magnification (B).

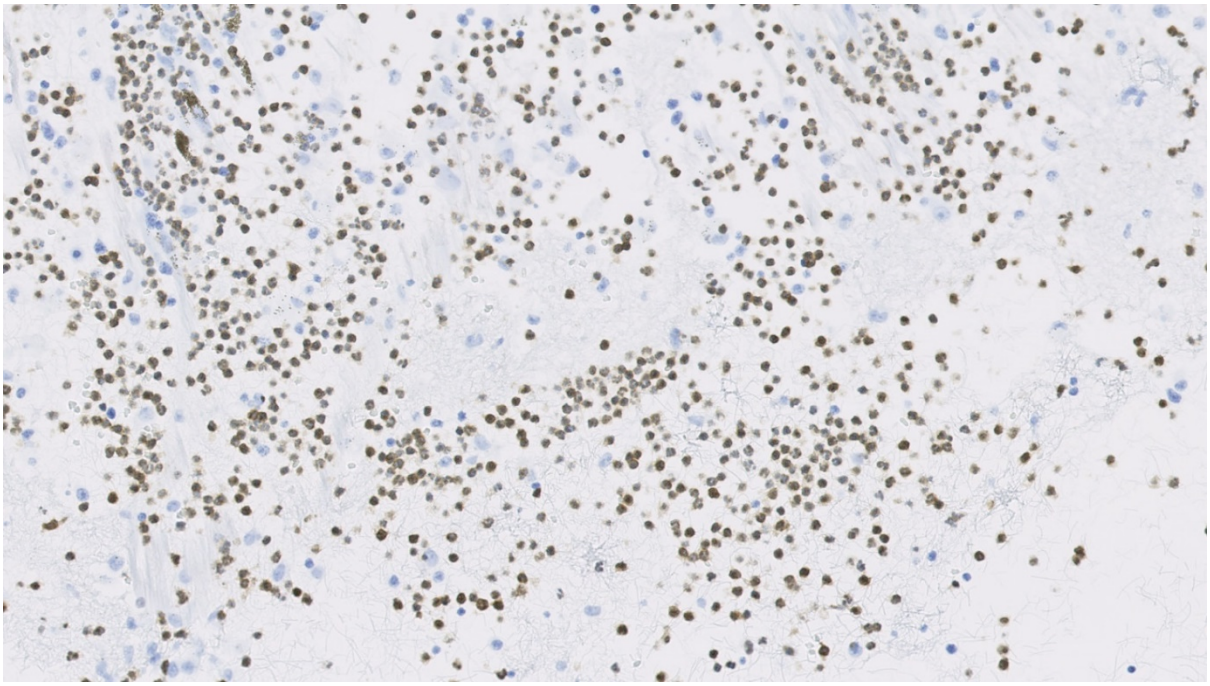

**Figure 2.18:** Case 17, calprotectin-positive cells in the vitreous cavity. There was a posterior neutrophilic phakitis with rupture of the posterior lens capsule. This was a case of *M. bovis* infection. x200 magnification.

## 2.4 Discussion

The data presented in this chapter show that cases of feline ocular mycobacteriosis typically present histologically with granulomatous to pyogranulomatous chorioretinal lesions, retinal detachment, and photoreceptor atrophy. The distribution of these lesions mimic that observed in cases of ocular metastatic neoplasia in cats, where there is a tendency for lesions to present in the posterior uvea (Dubielzig *et al.*, 2010b). In a small number of cats, inflammatory cells were present across all three layers of the eye, resulting in an endophthalmitis. Inflammatory cells were also frequently seen surrounding and invading the optic nerve. Lesions affecting the anterior external ocular and periocular tissues *i.e.*, the cornea, conjunctiva, and anterior sclera, were less common and where inflammatory lesions were restricted to these tissues it was hypothesised this was due to direct penetration and inoculation rather than haematogenous dissemination of mycobacteria to the uvea. In some cases, there was a substantial number of infiltrating lymphocytes, particularly in the retina where these accumulated around blood vessels, and in the choroid where segmental infiltration of Pax5-positive B-cells was noted. Minimal to mild lymphoplasmacytic inflammation within the iris was also a common feature; this may have been attributable to mycobacterial infection, or it could reflect a non-specific inflammatory response or be due to an underlying unrelated pathological process such as idiopathic anterior uveitis. Over 90% of cases were due to infection with MTBC pathogens, of which *M. bovis* was the most frequently identified species (where a species-level diagnosis was available), and two cases of ocular *M. lepraemurium* infection were reported, which has previously been considered as not capable of causing ocular disease (O'Brien *et al.*, 2017c). Staining with ZN revealed the presence of AFB in 83% of cases, typically in regions of necrosis, and some cases of infection with MTBC pathogens presented with huge numbers of AFB. These findings would suggest that the immune response to ocular mycobacterial infection is broadly similar to what is recognised in cases of cutaneous and lymph node TB (see Chapter 3) (Kipar *et al.*, 2003, Mitchell *et al.*, 2021b), although there did appear to be greater recruitment of lymphocytes, in particular B-cells, in ocular lesions. Therefore, if a cat is presenting with acute-onset blindness, and choroidal lesions and retinal detachment are identified on fundoscopic examination, mycobacterial infection should be considered as a differential diagnosis, in particular infection with *M. bovis*. When taken together, given the acute onset presentation and the identification of AFB in most enucleated eyes, cats may not be an appropriate species to act as a model for naturally occurring cases of human OTB.

This chapter presents the largest study to date of the histopathological and immunohistochemical features of feline ocular mycobacterial lesions, greatly expanding our knowledge on this subject, and will provide a valuable resource for histopathologists, veterinary immunologists and comparative ophthalmologists. All of the cases examined showed extensive histopathological changes, which is consistent with the acute onset clinical presentation of feline ocular mycobacterial disease (Stavinohova *et al.*, 2019). This is in contrast with cases of human OTB, where more subtle clinical signs are recorded and cases typically present with low-grade chronic inflammatory lesions (Testi *et al.*, 2019, Petrushkin *et al.*, 2020). There may be a degree of bias, where owners do not recognise cases of feline ocular

mycobacteriosis until there are extensive changes; therefore, the histopathological changes observed in these eyes are much more pronounced than those seen in cases of human OTB. It may be that there is a spectrum of ocular disease, with progression from subtle changes in the choroid and retina which can affect vision, allowing for self-reporting of humans to their physician, and if left untreated these lesions develop into choroidal granulomas with secondary changes such as subretinal exudation, retinal detachment and potentially glaucoma (Dubielzig *et al.*, 2010a, Dubielzig *et al.*, 2010b, Stavinohova *et al.*, 2019). Given that approximately 6% of cases of feline mycobacterial disease have ocular lesions (Gunn-Moore *et al.*, 2011a), and these are typically advanced at the point of diagnosis, fundoscopic examination should be advised in cases of suspected or confirmed mycobacterial infection to try and identify lesions that may mirror the subtle signs documented in human OTB (Agrawal *et al.*, 2018).

A major finding of the work presented in this chapter was the identification of ZN-positive organisms in 83% of globes. This is contrast to cases of human OTB, where lesions are typically negative on ZN-staining for AFB (Wroblewski *et al.*, 2011). The lack of identification of AFB in human OTB lesions, and poor recovery of positive PCR results on aqueous or vitreous samples (Wroblewski *et al.*, 2011), has led some to believe that these lesions are purely inflammatory, resulting from antigenic mimicry between mycobacterial and retinal antigens (Garip *et al.*, 2009) or they are hypersensitivity reactions (Duke-Elder, 1965). As a result, it has been proposed that cases may respond to treatment with anti-inflammatory therapy alone. This is not the case in cats; in some cases of infection with MTBC pathogens there were large numbers of AFB, recorded as high grade BI scoring. Acid-fast bacilli were often found within areas of necrosis, but only one case with endophthalmitis had intra- and extracellular ZN-positive organisms within the vitreous cavity while no organisms were identified in the anterior chamber. Therefore, sampling of aqueous and/or vitreous humor may be extremely limited to assist in the diagnosis of cases of ocular mycobacteriosis in both humans and cats (Featherstone and Scurrell, 2015). As *M. bovis* was the most frequently identified species of mycobacteria causing ocular lesions, any cat presenting with retinal detachment and choroidal lesions should be treated as a suspected case of *M. bovis* infection, in order to help protect human health (Human Animal Infections and Risk Surveillance (HAIRS) group, 2014), and further investigations should be performed as necessary to rule in or out other causes of acute onset blindness and retinal detachment.

The eye is an immunoprivileged site and acts as a lymph node for cases of intra-ocular infection (Streilein, 2003, Maggs, 2009). Despite this, there is little data describing the normal immune cell composition within the feline eye (McMenamin, 1997), and knowledge of how the eye responds to infection can inform us on how best to diagnose, treat and manage such cases. Studies describing the histopathological and immunohistochemical features of (muco)cutaneous and lymph node feline TB lesions showed that the appearance of the granuloma could help differentiate between *M. bovis* and *M. microti* (see Chapter 3) (Mitchell *et al.*, 2021b). These descriptions of ‘classical’ and ‘atypical’ granulomas were found to translate to some ocular mycobacterial lesions, but there was a large proportion of cases where there was no clear structure to the (pyo)granulomatous inflammatory infiltrate; hence, this was termed ‘unstructured’. There were also substantial numbers of B-cells in many ocular lesions; B-cells are thought to play an immunomodulatory role in the pathogenesis of

mycobacterial infections (Rijnink *et al.*, 2021), and their involvement may contribute to the lack of granuloma formation in ocular lesions. Additionally, features of mycobacterial lesions such as collagen deposition forming a fibrous capsule around granulomas were recorded inconsistently. Features such as choroidal fibrosis and retinal gliosis could have a devastating impact on vision, therefore the eye serves to limit such responses to injury (Aguilar and Green, 1984, Cepko and Dyer, 2000), which may be why encapsulation of lesions was not seen in all cases. Further descriptions of the histopathological and immunohistochemical features of feline TB lesions are presented in the following chapter.

To summarise, feline ocular mycobacterial lesions are most frequently found in the choroid and retina, with extension to the surrounding tissues, predominantly consisting of epithelioid macrophages, but also with a substantial lymphoplasmacytic component in some cases. It is most likely infection of the eye occurs following haematogenous dissemination of mycobacteria from the primary site of infection, although direct inoculation of mycobacteria into the ocular tissues also occurs, and AFB morphologically consistent with mycobacteria can be found in most globes, sometimes in large numbers. These features differ from what has been reported in cases of human OTB; therefore, the cat may not be an accurate model for human OTB, but it may prove interesting for further comparative studies to understand how and why these host-species differences occur and develop.

# Chapter 3: Histological and Immunohistochemical Features Suggesting Aetiological Differences in Lymph Node and (Muco)cutaneous Feline TB Lesions

## Preface

The following chapter consists of one article published in the Journal of Small Animal Practice. This is an open access article under the terms of the Creative Commons Attribution License, which permits its use, distribution and reproduction in any medium, provided the original work is properly cited.

**Mitchell, J. L.**, Del Pozo, J., Woolley, C. S. C., Dheendsa, R., Hope, J. C. and Gunn-Moore, D. A. Histological and immunohistochemical features suggesting aetiological differences in lymph node and (muco)cutaneous feline tuberculosis lesions. *Journal of Small Animal Practice*. 2021; 1-14.

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## Author's Contribution

The author identified and selected the cases for inclusion, performed the histopathological and quantitative analysis, the statistical analysis and wrote the manuscript. The author performed H&E, ZN, and immunohistochemical staining for cases 6, 7, 8, 10, 12, 14, 17, 20 and 21. For cases 2, 4, 5, 11, 13, 15, 18 and 19 H&E and ZN staining was performed by C. S. C. Woolley (The Roslin Institute), and immunohistochemical staining was performed by the author. Cases 1, 3, 9, 15 and 22 were stained (H&E, ZN, IHC) by R. Dheendsa (Royal (Dick) School of Veterinary Studies) under the instruction and supervision of the author. For all cases MT staining was performed by the VPU.

## 3.1 Introduction

The previous chapter demonstrated that for less common presentations of feline mycobacteriosis a thorough understanding of the histopathological features of mycobacterial lesions can help diagnose these infections, as well as provide further understanding of the underlying immunopathogenesis and how this may be conserved or differ across species. Histopathology is often the first step towards attaining a diagnosis of mycobacterial infection in the cat (O'Halloran and Gunn-Moore, 2017), aided by special stains such as ZN or FF to detect AFB morphologically consistent with mycobacteria. While there are descriptions of the histopathological features of feline mycobacterial lesions (Malik *et al.*, 2002, Davies *et al.*, 2006, Gunn-Moore *et al.*, 2011b), comparing lesions between different species of mycobacteria is uncommon. This is particularly true for cases of TB, caused by *M. bovis* or *M. microti*. There are also very few studies describing the immunohistochemical features of such lesions (Kipar *et al.*, 2003, Peterhans *et al.*, 2020).

In GB, where specialist mycobacterial culture of lesions was successful, over 70% of cases of feline mycobacteriosis are due to infection with members of the MTBC, namely *M. bovis* and *M. microti* (Gunn-Moore *et al.*, 2011a). While both infections are treated with the same combination of antimicrobials (O'Halloran and Gunn-Moore, 2017), differences exist in the zoonotic potential of these mycobacterial species (Gunn-Moore, 2014). Reports of cases of cat-to-human transmission of mycobacteria are sporadic and rare (Gunn-Moore and Lalor, 2015, O'Connor *et al.*, 2019), estimated at six cases in the last 150 years, globally (O'Halloran and Gunn-Moore, 2017); all were associated with *M. bovis* infection. There may also be differences in the transmissibility of these pathogens; for example, a nosocomial outbreak of *M. bovis* has been reported (Murray *et al.*, 2015), whereas this has not been demonstrated for *M. microti*. Therefore, it is important to obtain a species-level diagnosis in cases of feline mycobacteriosis, to determine the risk posed to owners and veterinary staff when dealing with these cases. Currently, this can be achieved with either mycobacterial culture or PCR, but there are limitations to both methodologies, namely the duration of time it takes to obtain a culture-positive result as well as the poor sensitivity (Gunn-Moore *et al.*, 2011a), and PCR testing may be prohibitively expensive (O'Halloran and Gunn-Moore, 2017). Being able to provide owners and clinical staff with an informed suspicion of either of these pathogens at an earlier stage in the diagnostic work-up may assist with the decision-making process, as well as help to safeguard human health, especially if there is an immunocompromised individual in contact with the infected cat. Histopathology and/or IHC may provide a means by which this could be achieved.

Despite increasing awareness of mycobacterial infections in cats, very few studies have explored the histopathological and immunohistochemical features of such lesions (Kipar *et al.,* 2003, Peterhans *et al.*, 2020). Therefore, there is a deficiency in our knowledge of how the feline immune system responds to these infections, and whether there are broad similarities with mycobacterial infections in other species or if there are feline-specific idiosyncrasies, and if so, how, and why do these arise. Immunohistochemistry has been used to describe the cellular composition of mycobacterial lesions in

many species (Canfield *et al.*, 2002, Palmer *et al.*, 2007, García-Jiménez *et al.*, 2012, García-Jiménez *et al.*, 2013), as well as to investigate the expression of different cytokines (Pereira-Suárez *et al.*, 2006, Canal *et al.*, 2017), but these studies have not extended to compare cell populations between lesions caused by different members of the MTBC within the same species. Immunohistochemistry can be useful for the classification of neoplastic lesions such as T- and B-cell lymphomas in cats (Jackson *et al.*, 1996); therefore, if differences exist in the cell populations of lesions caused by infection with *M. bovis* or *M. microti* this could provide a rapid means to discern between infections with these two pathogens.

The aim of this chapter, presented in the following paper, was to determine whether histopathological or immunohistochemical features of feline TB lesions could be used to determine infection with either *M. bovis* or *M. microti*. A secondary aim was to provide further descriptions of both *M. bovis* and *M. microti* lesions in cats, as such information is lacking from the literature, and investigate whether TB lesion scoring systems established for use in cattle and expanded for use in other species are also applicable to cats (Wangoo *et al.*, 2005). Finally, a scoring system comprising of a BI and grade, adapted from one used in cases of human leprosy (Ridley, 1964), was used to enable an objective description of the bacterial burden within feline TB lesions.

PETSAVERS PAPER

# Histological and immunohistochemical features suggesting aetiological differences in lymph node and (muco) cutaneous feline tuberculosis lesions

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**Objectives:** To identify and describe histological and immunohistochemical criteria that may differentiate between skin and lymph node lesions associated with *Mycobacterium* (*M.*) *bovis* and *M. microti* in a diagnostic pathology setting.

**Materials and Methods:** Archived skin and lymph node biopsies of tuberculous lesions were stained with haematoxylin and eosin, Ziehl-Neelsen and Masson's Trichrome. Immunohistochemistry was performed to detect the expression of calprotectin, CD3 and Pax5. Samples were scored for histological parameters (*i.e.* granulomas with central necrosis versus small granulomas without central necrosis, percentage necrosis and/or multinucleated giant cells), number of acid-fast bacilli (bacterial index) and lesion percentage of fibrosis and positive immunohistochemical staining.

**Results:** Twenty-two samples were examined (*M. bovis* n=11, *M. microti* n=11). When controlling for age, gender and tissue, feline *M. bovis*-associated lesions more often featured large multi-layered granulomas with central necrosis. Conversely, this presentation was infrequent in feline *M. microti*-associated lesions, where small granulomas without central necrosis predominated. The presence of an outer fibrous capsule was variable in both groups, as was the bacterial index. There were no differences in intralesional expression of immunohistochemical markers.

**Clinical Significance:** Differences in the histological appearance of skin and lymph node lesions may help to infer feline infection with either *M. bovis* or *M. microti* at an earlier stage when investigating these cases, informing clinicians of the potential zoonotic risk. Importantly, cases of tuberculosis can present with numerous acid-fast bacilli. This implies that a high bacterial index does not infer infection with non-zoonotic non-tuberculous mycobacteria.

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## INTRODUCTION

Mycobacterial infections are increasingly recognised as a substantial cause of morbidity in the domestic cat population, especially in Great Britain (Broughan *et al.* 2013), where approximately 1% of all feline biopsy submissions show changes suggestive of mycobacterial disease (Gunn-Moore *et al.* 2013). The most common presentation of feline mycobacteriosis is raised cutaneous lesions; these may be ulcerated and discharging sinus tracts can be present. Local lymphadenitis is frequently identified, presumptively due to drainage of mycobacteria-laden immune cells, which are predominantly macrophages (Ganbat *et al.* 2016), from the cuta-





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neous lesion (Gunn-Moore *et al*. 2011a). This clinical presentation is recognised in cases of infection with members of the *Mycobacterium* (*M*.) *tuberculosis*-complex (MTBC) as well as non-tuberculous mycobacteria (NTM) such as members of the *M. avium*-complex (Gunn-Moore 2014). Infection can spread to the lungs, putatively due to haematogenous spread within monocytes and neutrophils (Latimer *et al*. 1997, Krishnan *et al*. 2010), resulting in a broncho-interstitial pulmonary pattern (Bennett *et al*. 2011). Mycobacterial lesions have also been reported in the eyes (Stavinohova *et al*. 2019), joints (Lalor *et al*. 2017) and other organs and tissues, resulting in a range of clinical signs (Gunn-Moore 2014).

The MTBC consists of 10 different mycobacterial species (Rodriguez-Campos *et al*. 2014, Dippenaar *et al*. 2015), responsible for causing tuberculosis (TB) across a wide taxon of animals. Those that cause TB in the domestic cat are *M. bovis* and *M. microti*, the vole bacillus (Gunn-Moore *et al*. 2011a), both of which are potentially zoonotic (Emmanuel *et al*. 2007, O'Connor *et al*. 2019). While *M. bovis* has been identified as causing disease across multiple species of veterinary interest, reports of *M. microti* infection in species other than the domestic cat and its maintenance host the field vole (*Microtus agrestis*) are uncommon, although they are being identified more frequently (Smith *et al*. 2009, Boniotti *et al*. 2014, Michelet *et al*. 2015). Where positive culture results were obtained, over 70% of feline mycobacterial infections in Great Britain were due to MTBC pathogens, fairly evenly distributed between *M. microti* and *M. bovis* (approximately 40% and 33%, respectively) (Gunn-Moore *et al*. 2011a). These appear to localise to specific regions of the UK; *M. bovis* is more frequent in cats from TB endemic areas, whereas *M. microti* infection is more frequent in cats from areas with low or null prevalence of bovine TB (*i.e*. Scotland), (Burthe *et al*. 2008).

*M. bovis* and *M. microti* infections produce identical macroscopic lesions; however, they differ in their zoonotic potential, and for some owners the decision to treat or euthanise their cat can depend on whether *M. bovis* is demonstrated as the infective organism (O'Halloran & Gunn-Moore 2017). Therefore, the ability to rapidly discern between the two causative agents of feline TB is essential and can inform the potential risk posed to owners as well as veterinary staff (de la Rua-Domenech 2006).

Histopathology is often the first diagnostic test performed when investigating a potential case of mycobacterial disease in cats (Gunn-Moore 2014, O'Halloran & Gunn-Moore 2017), and lesions featuring granulomatous to pyogranulomatous inflammation, dominated by epithelioid macrophages, raise the suspicion index of mycobacterial disease (Gunn-Moore *et al*. 2011b). Unlike in other species, multinucleated giant cells (MNGCs) are not routinely observed in feline mycobacterial lesions (Kipar *et al*. 2003). Special stains, such as Ziehl-Neelsen (ZN), can be performed on sections where granulomatous to pyogranulomatous inflammation is identified to confirm the presence of acid-fast bacilli (AFB) morphologically consistent with mycobacteria. However, many feline mycobacterial lesions are paucibacillary, *i.e*. they have few to no obvious AFB on ZN-staining, so a negative result does not rule out mycobacteriosis (Gunn-Moore *et al*. 2013). Abundant numbers of AFB, where identified, have been associated with cases of lepromatous feline leprosy, a disease entity caused by non-zoonotic NTM species (Malik *et al*. 2002, O'Brien *et al*. 2017).

Subsequent diagnostic testing for feline mycobacterial infections can be challenging. Specialist culture, which is the validated assay, has poor sensitivity (Gunn-Moore *et al*. 2011a) and it can take at least 3 months to obtain a positive result for slow-growing mycobacterial species, which includes *M. microti* (Smith *et al*. 2009). More rapid testing methodologies are available, including molecular-based diagnostics, such as polymerase chain reaction (PCR) assays (Aranaz *et al*. 1996, Richter *et al*. 2003); however, they are less sensitive on formalin-fixed tissues compared to fresh samples (Reppas *et al*. 2013). Recent advancements in molecular biology have improved the ability of tests to discriminate between members of the MTBC, resulting in an increase in the identification of *M. microti* as a causative agent of TB lesions across a range of species (Boniotti *et al*. 2014, Michelet *et al*. 2015, Landolt *et al*. 2019, Pérez De Val *et al*. 2019). Despite this, the financial constraints of owners may limit the use of these techniques for the species-level diagnosis of feline mycobacterial infections. The interferon-gamma (IFN$\gamma$) release assay (IGRA) has excellent sensitivity for detecting MTBC infections and is reasonable at differentiating between *M. bovis* and *M. microti*; however, financial constraints may still apply, obtaining sufficient volumes of blood may be a limiting factor in some cats, and the test requires careful handling and processing of heparinised blood (Rhodes *et al*. 2008, Rhodes *et al*. 2011).

The hallmark of TB is granulomatous inflammation; this term can encompass a range of histopathological presentations, from unorganised macrophage infiltration, to the presence of well-formed granulomas (Shah *et al*. 2017). A precise definition for what constitutes a granuloma is difficult, but at its simplest, the granuloma can be thought of as "an organised collection of mature mononuclear phagocytic cells" (Adams 1976, Pagán & Ramakrishnan 2018). Tuberculous granulomas typically consist of epithelioid macrophages surrounding a necrotic core, encapsulated by fibroblasts and an outer layer of lymphocytes (Martin *et al*. 2016). The tuberculous granuloma within lymph nodes has been well described in cattle (Wangoo *et al*. 2005) and these histological findings have been applied to a number of other livestock (Sanchez *et al*. 2011, Vallejo *et al*. 2018) and wildlife species (Canfield *et al*. 2002, García-Jiménez *et al*. 2012, García-Jiménez *et al*. 2013).

The granuloma can be characterised further by its cellular composition using immunohistochemistry (IHC). This has been performed extensively in both experimental and natural *M. bovis* infections of livestock (Pereira-Suárez *et al*. 2006, Palmer *et al*. 2007, Sanchez *et al*. 2011, Canal *et al*. 2017, Vallejo *et al*. 2018) and wildlife species (Canfield *et al*. 2002, García-Jiménez *et al*. 2012, García-Jiménez *et al*. 2013), as well as one study describing *M. microti* lesions in its natural host, the field vole (Kipar *et al*. 2014). One study has been performed on feline mycobacterial lesions (Kipar *et al*. 2003); however, this did not explore differences between *M. bovis* and *M. microti* infections.


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The objective of the current study was to describe and compare the histological and immunohistochemical features of feline tuberculous granulomas in (muco)cutaneous and lymph node lesions to identify potential patterns that help differentiate between the two causes of TB in cats. We hypothesise that the nodal/(muco)cutaneous granulomas observed in cats infected with *M. microti* differ in structural organisation and immune cell populations compared to those in *M. bovis*-positive cats.

## MATERIALS AND METHODS

### Animals and samples

Ethical approval for this study was granted by the institutional Veterinary Ethical Review Committee (approval no. 79 14).

A database maintained on Microsoft Excel © 2016 (Microsoft Corporation) by an independent researcher was searched by the lead investigator in May 2019 to identify cats where a formalin-fixed paraffin-embedded (FFPE) tuberculous (muco)cutaneous or lymph node lesion biopsy had been submitted to the institution by referring veterinary surgeons (RVS) and commercial histopathology laboratories following owner consent, and that had a culture, PCR or IGRA diagnosis of *M. bovis* or *M. microti* infection. (Muco)cutaneous biopsies were taken to include those recorded as being "skin," "dermal," "subcutaneous" and "gum". The biopsies had been taken as part of the diagnostic investigation of these cases or at *post-mortem* examination and were fixed in 10% neutral buffered formalin for a minimum of 24 hours post-sampling and processed for histology using standard methods. Anti-mycobacterial therapy had not been given before sampling. Mycobacterial disease was suspected based on histopathological examination and/or ZN-staining, and subsequent speciation was confirmed by external specialist mycobacterial culture (Animal and Plant Health Agency), PCR (Leeds Teaching Hospitals NHS Trust) or IGRA (Biobest Laboratories). An IGRA diagnosis of *M. microti* was considered definitive if the IGRA result showed a biased response to purified protein derivative (PPD) from *M. bovis* (PPDB) over PPD from *M. avium* (PPDA) and no response to the antigenic cocktail of early secretory antigenic target 6 kDa (ESAT-6)/culture filtrate protein 10 kDa (CFP-10) (Rhodes *et al.* 2011), the cats had lesions exhibiting histological features consistent with mycobacterial infection, and they lived in regions of England that are low-risk areas for bovine TB or in Officially TB Free regions, *i.e.* Scotland. Conversely, an IGRA diagnosis of *M. bovis* was considered definitive if the IGRA result was PPDB-biased over PPDA and ESAT-6/CFP-10 was either positive or negative (Rhodes *et al.* 2011), with histological features consistent with mycobacteriosis, and the cat had been living in an area with bovine TB. The exception to having been living in a "high-risk" or "edge" area for bovine TB, is any cat fed the raw commercial diet that has recently been associated with an outbreak of *M. bovis* in cats in England and Scotland (O'Halloran *et al.* 2019). Eligible cases were cross-referenced with clinical histories supplied by the RVS to identify additional clinical information, *i.e.* results of testing for feline leukaemia virus (FeLV) p27 antigen and feline immunodeficiency virus (FIV) anti-p24 antibodies (SNAP FIV/FeLV Combo Test, Idexx). For each cat, a single FFPE tissue block was selected; serial sections were prepared for histopathology and IHC.

Control FFPE tissues for IHC were a submandibular lymph node from a cat euthanased for reasons not related to mycobacterial infection, and a cutaneous lesion from a cat diagnosed with a NTM infection on IGRA with abundant AFB.

### Histopathology

Histopathology was performed to assess the morphological features of the mycobacterial lesions (henceforth, the word lesion will always refer to a granulomatous or pyogranulomatous inflammatory infiltrate). Four-micron thick sections were cut and stained with haematoxylin and eosin (H&E), Ziehl-Neelsen (ZN) and Masson's Trichrome (MT). H&E-stained slides were evaluated to describe the cellular composition and granuloma type of the lesions, with a focus on specific parameters.

A lesion was defined as an area of infiltration by macrophages which were mostly epithelioid. The histological presentation of lesions was assessed and compared to previously described classification systems in other species (Wangoo *et al.* 2005). However, these systems did not adapt to feline tuberculous lesions, as MNGCs and dystrophic mineralisation were not present in any case. For this reason, a novel, description-based classification was devised for this study (see results). Necrosis was defined as areas with loss of cellular and structural detail, with accumulation of eosinophilic and basophilic (karyorrhectic) debris. This presentation is consistent with caseous necrosis (Miller & Zachary 2017).

Slides stained with ZN were examined under standard light microscopy to determine the bacterial index (BI). To do this, the number of individual AFB were counted in 15 randomly chosen high-power fields (hpf) (×1000 magnification) and an average was calculated (AFB/hpf). A modified Ridley BI score, used for leprosy in humans (Ridley 1964), was used to grade the number of AFB/hpf on a scale of 0 to 6: 0=no AFB/hpf; 1=0.01 to 0.1; 2=0.11 to 1.0; 3=1.01 to 10; 4=10.01 to 100; 5=100.01 to 1000; 6=>1000 AFB/hpf). In accordance with the Ridley BI score, those graded 0 to 2 were categorised as low BI and those 3 to 6 as high BI.

Slides stained with MT, to identify collagen, were analysed as described below to obtain the lesion percentage value of fibrosis.

### Immunohistochemistry

IHC was performed to detect the expression of calprotectin, CD3 and Pax5. Briefly, sections were mounted on SuperFrost® Plus-coated slides (Thermo Electron Ltd.), dewaxed, rehydrated, rinsed in distilled water and then washed in Tris-buffered saline (TBS) with Tween®20 (TBS-T) (28,360, Thermo Scientific) before antigen-retrieval. All antibodies were diluted in TBS-T and all washes between steps were in TBS-T. For calprotectin, which is expressed by granulocytes, monocytes and recently blood-derived macrophages (Rugtveit *et al.* 1996), mouse monoclonal anti-human macrophages antibody, clone MAC387 (MCA874G, Bio-Rad) (Kipar *et al.* 2003) was diluted to a final concentration of 1/800 (1.25 µg/mL) and incubated for 30 minutes at room temperature (RT) follow-

ing epitope retrieval using proteinase K (S302080, Dako) for 20 minutes at RT. For CD3, a pan-T-lymphocyte marker, rabbit polyclonal anti-human CD3 antibody (A045201, Dako) (Kipar *et al*. 2003) was diluted 1/100 and incubated for 30 minutes at RT following epitope retrieval using 0.01 M sodium citrate buffer, pH 6.0 at 121°C for 40 minutes. Heat-induced epitope-retrieval (HIER) was performed using a SES Little Sister 3 autoclave (Eschmann). For Pax5 (a.k.a B-cell-specific activator protein [BSAP]) which is expressed by all stages of B-lymphocytes, although not plasma cells (Barberis *et al*. 1990), mouse monoclonal anti-human BSAP antibody, clone DAK-Pax5 (M730701) (Agostinelli *et al*. 2010) was diluted 1/50 (3.14 µg/mL) and incubated for 30 minutes at RT following HIER as for CD3. After incubation with the primary antibody, non-specific endogenous peroxidase activity was blocked using REAL Peroxidase-Blocking Solution (S202386, Dako) for 10 minutes at RT. Sections were then incubated with goat anti-mouse/anti-rabbit secondary detection polymer (EnVision™ + Dual Link System-HRP, K406311, Dako) for 45 minutes at RT, followed by visualisation of positive staining with 3,3′-diaminobenzidine tetrahydrochloride (DAB) for 10 minutes at RT. Slides were counterstained with haematoxylin and Scott's Tap Water for 10 seconds each, then dehydrated, cleared in xylene, mounted and a cover-slip was added.

Positive controls were feline tissues known to express the antigen of interest (calprotectin: an NTM-infected cutaneous lesion; CD3 and Pax5: a normal lymph node). An isotype control was used to assess non-specific staining for monoclonal antibodies, using an isotype and concentration-matched mouse anti-chicken Bu-1a/b, clone AV20 (MCA5764, Bio-Rad). Negative controls were run with omission of the primary antibody.

### Image analysis

All H&E, MT and immunolabelled-slides were scanned with a NanoZoomer-XR scanner, using the NDP.scan Ver.3.2.12 software (Hamamatsu Photonics) and images viewed on NDP.view2 Ver.2.7.52 (Hamamatsu Photonics) to assess the cellular populations and granuloma type of the (pyo)granulomatous infiltrate. Quantification of necrosis and the total lesion area was determined using QuPath Ver.0.1.2 (Bankhead *et al*. 2017), with each region of interest (ROI) outlined manually using the drawing tools and the ROI file saved. To quantify the amount of fibrosis (in MT-stained sections), and the area of positive immunostaining (in IHC-stained sections), an adaptation of previously reported methods was used, using image deconvolution (Chen *et al*. 2017). Briefly, the ROI for each tissue was delineated in QuPath and sent to ImageJ (Schneider *et al*. 2012). The outside of the image was cleared, the resultant file saved and then opened in FIJI (Schindelin *et al*. 2012). For MT-stained slides, the inbuilt parameters for "Alcian Blue & H" in the colour deconvolution plug-in gave the best separation of the image file into different colour channels. The threshold for positive fibrosis staining was set as the average auto-threshold (default) for each image file; an ImageJ macro was written to quantify the area of positive staining and the total lesion area. For immunolabelled sections the "H DAB" values of the above plug-in were used and the threshold for positive staining set as the auto-threshold value for the positive control slide. ImageJ macros were then written to calculate the positive staining and total lesion areas. Isotype and negative control slides were analysed using this threshold to calculate corrections in the TB slides for any non-specific staining. All image files were visually assessed to determine accuracy of the automated analysis. Macros written for image analysis are available as Supplemental Data 1.

### Statistical analysis

Data were analysed using RStudio (RStudio Team 2018). Univariate binomial logistic regression was performed to identify variables that may predict infection with either *M. bovis* or *M. microti*. Variables explored were age, gender, tissue, BI category, presence/absence of small granuloma clusters, necrosis, fibrosis, and expression of calprotectin, CD3 and Pax5. The proportion of necrosis, fibrosis and positive immunolabelling was converted to an ordinal scale variable. As age, gender and tissue were possible confounders, they were included as covariates for univariate analysis of BI category, granuloma type, necrosis, fibrosis, calprotectin, CD3 and Pax5. Multivariate logistic regression was subsequently performed on variables found to be significant on univariate analysis at $P < 0.20$, as well as the potential confounders age, gender and tissue. Variables were explored for collinearity and removed from the multivariate model where appropriate. Statistical significance for multivariate regression was set at $P < 0.05$.

## RESULTS

### Study population characteristics

Seventeen cats with *M. bovis* infection and 16 with *M. microti* infection with FFPE tissues available were identified from the database and assessed for eligibility. Six *M. bovis*-positive cats and two with *M. microti* were excluded as there was insufficient tissue in the FFPE block for further evaluation. Three cats diagnosed with *M. microti* on IGRA were identified as coming from high-risk areas for bovine TB and were subsequently excluded. Therefore, a total of 22 (*M. bovis* n=11, *M. microti* n=11) archived FFPE (muco)cutaneous and lymph node biopsies of tuberculous lesions were selected; they came from throughout England and Scotland. Further details are available in Table 1.

Nearly 60% (13/22) of the cats in this study were male, and all cats were neutered. The median age of cats infected with *M. bovis* was 5 years, 1 month, compared to 7 years, 0 months for *M. microti*-infected cats; this difference in age was not statistically significant [P=0.326, odds ratio (OR)=0.99 (0.97 to 1.01)]. Eighty-six percent of cats (19/22) were either domestic short or long-haired cats. Other than case 1, all cats were reported as being hunters, or having outdoor access. Case 1 was an indoor-only cat with no reported contact with potentially infected wildlife; however, it was fed the commercial raw food diet associated with the outbreak of *M. bovis* in cats in England and Scotland in 2018 to 2019 (O'Halloran *et al*. 2019). Infection with FeLV or FIV was not identified in the five cats that were tested.

**Table 1. Summary of animal details included in this study**

| Case | Diagnosis | Method | Age | Gender | Breed | Location | FeLV antigen | FIV antibody | Site of Lesion |
|---|---|---|---|---|---|---|---|---|---|
| 1 | *M. bovis* | Culture | 1year 0 month | MN | Persian | Greater Manchester | NP | NP | Mesenteric LN |
| 2 | *M. bovis* | Culture | 1year 7 months | FN | DSH | Bristol | NP | NP | Mesenteric LN |
| 3 | *M. bovis* | Culture | 1year 11 months | FN | DSH | Bristol | NP | NP | Inguinal mass |
| 4 | *M. bovis* | Culture | 3years 0 month | FN | DSH | Shropshire | Negative | Negative | Popliteal LN |
| 5 | *M. bovis* | Culture | 4years 0 month | MN | DSH | Wiltshire | NP | NP | Submandibular LN |
| 6 | *M. bovis* | PCR | 5years 1 month | FN | DSH | East Sussex | NP | NP | Axillary mass |
| 7 | *M. bovis* | Culture | 5years 4 months | MN | DSH | Bristol | NP | NP | Flank mass |
| 8 | *M. bovis* | Culture | 7years 0 month | FN | DSH | Dorset | NP | NP | Sinonasal mass |
| 9 | *M. bovis* | PCR | 8years 0 month | FN | DSH | Somerset | NP | NP | Pedal mass |
| 10 | *M. bovis* | Culture | 10years 3 months | MN | DLH | Staffordshire | NP | NP | Inguinal mass |
| 11 | *M. bovis* | IGRA | 12years 1 month | MN | DSH | Shropshire | Negative | Negative | Popliteal LN |
| 12 | *M. microti* | Culture | 1year 5 months | MN | DSH | Essex | Negative | Negative | Gingival mass |
| 13 | *M. microti* | IGRA | 2years 0 month | MN | DSH | Fife | NP | NP | LN (unspecified) |
| 14 | *M. microti* | IGRA | 5years 4 months | MN | DSH | Angus | NP | NP | Forelimb mass |
| 15 | *M. microti* | IGRA | 6years 6 months | FN | DSH | Perth & Kinross | NP | NP | Leg mass |
| 16 | *M. microti* | IGRA | 6years 10 months | FN | DSH | Lancashire | NP | NP | LN (unspecified) |
| 17 | *M. microti* | Culture | 7years 0 month | MN | Siamese | London | Negative | Negative | Flank mass |
| 18 | *M. microti* | Culture | 7years 0 month | FN | DSH | Angus | NP | NP | Skin (unspecified) |
| 19 | *M. microti* | Culture | 8years 0 month | MN | Burmilla | East Lothian | NP | NP | Mesenteric LN |
| 20 | *M. microti* | Culture | 8years 0 month | MN | DSH | Kent | NP | NP | Facial mass |
| 21 | *M. microti* | Culture | 8years 10 months | MN | DSH | Devon | NP | NP | Facial mass |
| 22 | *M. microti* | IGRA | 15years 0 month | MN | DSH | Kent | Negative | Negative | Dorsal mass |

PCR Polymerase chain reaction, IGRA Interferon-gamma release assay, MN Male neutered, FN Female neutered, DSH Domestic shorthair, DLH Domestic longhair, FeLV Feline leukaemia virus, FIV Feline immunodeficiency virus, NP Not performed, LN Lymph node
Cats were tested for FeLV p27 antigen and FIV anti-p24 antibodies using the SNAP FIV/FeLV Combo Test, Idexx

## Histopathological classification

Summarised results of histological and IHC analysis are presented in Table 2. Results of univariate analysis are reported in Table 3.

The lesions were granulomatous or pyogranulomatous, with macrophages as the predominant cell type in all cases, which were mostly epithelioid. Neutrophils were often present and were either dispersed throughout the lesion or accumulated in areas of necrosis. Lymphocytes were less frequently identified and were often located towards the periphery of lesions. Multinucleated giant cells were not identified in any sample.

A proportion of lesions of both aetiologies (10/22) were similar in appearance to typical tuberculous granulomas (Martin *et al*. 2016, Pagán & Ramakrishnan 2018), and these were termed "*organised*" granulomas (Fig 1A, B). "Organised" lesions had singular or multifocal zones of central caseous necrosis, surrounded by a layer of macrophages and epithelioid macrophages; neutrophils were also a common feature within this cell layer. Lymphocytes and plasma cells, where present, were often located towards the periphery of the lesions, and/or less often distributed throughout the lesion (where they occasionally clustered). There was inter-individual variation in lesion encapsulation, and an outer layer of concentrically arranged spindle-shaped cells admixed with eosinophilic fibrillary material (fibrous capsule), which ranged from a thick capsule surrounding the entire granuloma to no encapsulation at all. Where no obvious capsule was present, the inflammatory infiltrate extended into surrounding tissue.

The remaining lesions (12/22) had minimal to absent central necrosis. Clusters of epithelioid and non-epithelioid macrophages were present, with interspersed neutrophils and variable numbers of lymphocytes. These lesions were termed "*atypical*" granulomas (Fig 1C, D). Importantly, "atypical" lesions presented with well defined, mostly round, small epithelioid macrophage clusters (100 µm-1000 µm), often divided by thin fibrous septa into smaller clusters, with very rare and mild central necrosis. The presence of a well-defined outer fibrous capsule was variable, as for "organised" lesions.

Both granuloma types effaced and expanded pre-existing tissue and presented with frequent areas of collagen degeneration with increased intercellular spacing (oedema), with or without inflammation, as well as variable demarcation.

Eight of 11 *M. bovis*-associated lesions were classified as "organised," compared with two of 11 *M. microti*-associated lesions; the remaining three and nine lesions were deemed "atypical" for each aetiology, respectively. There was a statistically significant difference between aetiology and granuloma type in univariate logistic regression [P=0.024, OR=0.08 (0.01 to 0.73)], with "organised" lesions being more likely to arise from infection with *M. bovis*.

## Necrosis and fibrosis

Necrosis, as defined previously, was identified in 11 of the 22 cases examined, with larger necrotic areas in lesions with "organised" granulomas (these lesions had central necrosis, as described above). Most "atypical" tuberculous granulomas did not have any central necrosis, but clusters of necrotic neutrophils were identified in case 20 (Fig 1E). The median percentage area of necrosis

**Table 2. Summarised details of histological and immunohistochemical analysis of feline tuberculous lesions**

| Case | Aetiology | Granuloma t-type | Average AFB/hpf | BI score | BI grade | % Necrosis | % Fibrosis | % Calprotectin | % CD3 | % Pax5 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *M. bovis* | "Organised" | 63.40 | 4 | H | 50.8 | 28.3 | 20.5 | 0.1 | <0.1 |
| 2 | *M. bovis* | "Organised" | 22.20 | 4 | H | 58.9 | 1.2 | 5.6 | 0.4 | <0.1 |
| 3 | *M. bovis* | "Atypical" | 8.87 | 3 | H | 0.0 | 46.6 | 13.5 | 0.1 | <0.1 |
| 4 | *M. bovis* | "Organised" | 0.53 | 2 | L | 18.3 | 0.7 | 30.0 | 3.2 | 0.2 |
| 5 | *M. bovis* | "Organised" | 5.47 | 3 | H | 51.3 | 3.2 | 9.7 | 0.2 | 0.1 |
| 6 | *M. bovis* | "Organised" | 34.40 | 4 | H | 18.4 | 1.0 | 19.1 | 6.7 | 0.6 |
| 7 | *M. bovis* | "Atypical" | 0.93 | 2 | L | 0.0 | 23.8 | 22.2 | 8.5 | 0.3 |
| 8 | *M. bovis* | "Organised" | 0.33 | 2 | L | 6.9 | 10.5 | 10.4 | 0.8 | 0.1 |
| 9 | *M. bovis* | "Organised" | 11.47 | 4 | H | 27.0 | 8.5 | 16.1 | 4.3 | 0.8 |
| 10 | *M. bovis* | "Organised" | 15.60 | 4 | H | 32.0 | 33.8 | 14.2 | 8.4 | 0.5 |
| 11 | *M. bovis* | "Atypical" | 0.07 | 1 | L | 0.0 | 16.2 | 13.8 | 5.7 | 0.4 |
| 12 | *M. microti* | "Atypical" | 172.93 | 5 | H | 0.0 | 2.5 | 8.7 | 1.6 | 0.2 |
| 13 | *M. microti* | "Atypical" | 0.00 | 0 | L | 0.0 | 1.1 | 15.9 | 0.6 | <0.1 |
| 14 | *M. microti* | "Organised" | 0.47 | 2 | L | 28.4 | 35.5 | 32.2 | 1.7 | <0.1 |
| 15 | *M. microti* | "Atypical" | 0.27 | 2 | L | 0.0 | 8.8 | 9.7 | 0.3 | 0.1 |
| 16 | *M. microti* | "Atypical" | 0.00 | 0 | L | 0.0 | 13.2 | 3.9 | 1.3 | <0.1 |
| 17 | *M. microti* | "Atypical" | 1.40 | 3 | H | 0.0 | 12.9 | 28.1 | 6.9 | 0.7 |
| 18 | *M. microti* | "Atypical" | 0.33 | 2 | L | 0.0 | 10.0 | 12.1 | 6.5 | 0.1 |
| 19 | *M. microti* | "Atypical" | 0.07 | 1 | L | 0.0 | 4.6 | 11.1 | 0.3 | <0.1 |
| 20 | *M. microti* | "Atypical" | 0.20 | 2 | L | 9.3 | 3.8 | 18.8 | 1.3 | 0.1 |
| 21 | *M. microti* | "Organised" | 42.60 | 4 | H | 8.6 | 24.3 | 11.7 | 10.3 | 0.7 |
| 22 | *M. microti* | "Atypical" | 0.27 | 2 | L | 0.0 | 31.0 | 21.7 | 0.2 | 0.1 |

AFB Acid-fast bacilli, hpf High power field, BI Bacterial index, H High, L Low
Granuloma type was classified as either "organised" or "atypical." "Organised" granulomas consisted of a central necrotic core surrounded bey a cellular layer composed of epithelioid macrophages, macrophages and neutrophils. "Atypical" granulomas lacked a central necrotic core and consisted of tight aggregates of epithelioid macrophages, macrophages and neutrophils. In both granuloma types, lymphocytes were predominantly located at the periphery of the granuloma, and there was variable deposition of collagen fibres forming an external capsule in "organised" granulomas, or thin fibrous septa in "atypical" granulomas. "% Necrosis" and "% Fibrosis" is the area of each histological feature relative to the total area of the lesion, as determined on Haematoxylin & Eosin-, and Masson's Trichrome-stained slides. "% Calprotectin," "% CD3" and "% Pax5" is the area of positive staining for each marker relative to the total area of the lesion. Positive staining for calprotectin indicates the presence of granulocytes and recently blood-derived monocytes, CD3 for T-lymphocytes and Pax5 for B-lymphocytes

**Table 3. Results of univariate binomial logistic regression analysis**

| Variable | Summary statistics [median (range)] | | Odds ratio | 95% CI | P-value |
|---|---|---|---|---|---|
| | *M. bovis* | *M. microti* | | | |
| Granuloma type | A: 3, O: 8 | A: 9, O: 2 | 0.08 | 0.01 to 0.73 | 0.024 |
| Necrosis | 18.42% (0.00 to 58.90%) | 0.00% (0.00 to 28.35%) | | | |
| 0.00% | 3 | 8 | Baseline | — | — |
| 0.01 to 10% | 3 | 2 | 3.76 | 0.30 to 47.16 | 0.305 |
| >10% | 5 | 1 | 17.11 | 1.09 to 268.31 | 0.043 |
| BI category | H: 7, L: 4 | H: 3, L: 8 | 0.14 | 0.01 to 1.35 | 0.088 |
| Pax5 | 0.16% (<0.01 to 0.83%) | 0.07% (<0.01 to 0.72%) | | | |
| 0.00 to 0.09% | 4 | 6 | Baseline | — | — |
| 0.10 to 0.50% | 4 | 3 | 4.46 | 0.36 to 54.83 | 0.243 |
| >0.50% | 3 | 2 | 8.91 | 0.35 to 228.43 | 0.186 |
| Gender | MN: 5, FN: 6 | MN: 8, FN: 3 | 0.31 | 0.05 to 1.85 | 0.200 |
| Fibrosis | 10.54% (0.69 to 46.57%) | 9.95% (1.12 to 35.46%) | | | |
| 0.00 to 10% | 5 | 6 | Baseline | — | — |
| 10.01 to 20% | 2 | 2 | 2.00 | 0.13 to 31.62 | 0.623 |
| 20.01 to 30% | 2 | 1 | 7.64 | 0.31 to 187.52 | 0.213 |
| >30% | 2 | 2 | 4.48 | 0.19 to 106.37 | 0.353 |
| Calprotectin | 14.15% (5.57 to 30.05%) | 12.09% (3.91 to 32.15%) | | | |
| 0.00 to 10% | 2 | 3 | | | |
| 10.01 to 20% | 6 | 5 | 3.78 | 0.35 to 45.14 | 0.294 |
| >20% | 3 | 3 | 3.96 | 0.22 to 71.26 | 0.350 |
| Age | 5y 1m (1y 0m to 12y 1m) | 7y 0m (1y 5m to 15y 0m) | 0.99 | 0.97 to 1.01 | 0.326 |
| CD3 | 3.20% (0.07 to 8.49%) | 1.30% (0.17 to 10.32%) | | | |
| 0.00 to 1% | 5 | 4 | Baseline | — | — |
| 1.01 to 5% | 2 | 4 | 0.36 | 0.03 to 3.88 | 0.402 |
| >5% | 4 | 3 | 2.25 | 0.18 to 27.65 | 0.528 |
| Tissue | Skin: 6, LN: 5 | Skin: 8, LN: 3 | 0.69 | 0.12 to 3.78 | 0.665 |

BI Bacterial Index, A "Atypical", O "Organised", H High, L Low, MN Male neutered, FN Female neutered, y Years, m Months, LN Lymph node, CI Confidence interval
Age, gender and tissue were included as covariates for univariate regression of Necrosis, BI Category, Pax5, Calprotectin and CD3. Necrosis, Pax5, Calprotectin and CD3 P-values were calculated using the lowest group classification as the reference group. Goodness-of-fit analysis showed insufficient evidence to conclude the models did not fit the data (data not shown)

within *M. bovis* lesions was 18.3% (0.0 to 59.8%), compared to 0.0% (0.0 to 28.3%) for *M. microti* lesions. This was reflected in the results of univariate logistic regression, where if more than 10% of the lesion consisted of necrotic tissue, *M. bovis* was significantly more likely to be the organism associated with the lesion [P=0.043, OR=17.11 (1.09 to 268.31)].

In terms of fibrosis, there appeared to be qualitative differences in the distribution of collagen fibres within feline TB lesions; *M. bovis* lesions showed thicker bands of collagen fibres surrounding larger "organised" granulomas, whereas *M. microti* lesions displayed thinner fibrous septa dividing smaller "atypical" granulomas (Fig 1F). However, the total proportion of the lesion occupied by collagen fibres did not differ between aetiologies in univariate analysis (P > 0.213).

## Acid-fast bacilli counts and bacterial index category

AFB were identified in the ZN-stained section on examination of 15 hpf in 91% (20/22) of the samples. For the remaining two samples, the entire slide was examined to look for AFB; a single ZN-positive organism morphologically consistent with mycobacteria was identified in case 16, whereas no AFB were identified in case 13. Fifty-five percent of specimens scored as low BI (12/22); there was no statistical difference between the number of *M. bovis* and *M. microti* lesions scoring as low BI (P=0.088, OR=0.14 [0.01 to 1.35]). These data underline the variability in the number of organisms seen in feline tuberculosis lesions (*M. bovis* median: 8.87, range: 0.07 to 63.40; *M. microti* median: 0.27, range: 0.00 to 172.93). Case 12 scored BI 5, displaying vast numbers of AFB (Fig 2), often with an S-shaped morphology, and there were x1000 microscope fields from five other cats (cases 1, 2, 6, 9 and 21) with over 100 AFB (data not shown). ZN-positive organisms were found both intra- and extracellularly. Extracellular AFB were often located within areas of necrosis for both pathogens.

## Calprotectin immunohistochemistry

Calprotectin-positive cells, *i.e.* granulocytes, monocytes and recently blood-derived macrophages, with strong granular to diffuse cytoplasmic staining were present in all samples; for each case calprotectin had more percentage-lesion positive immunolabelling than CD3 and Pax5. In lesions with large areas of necrosis, calprotectin-positive cells were common at the cell-necrosis interface, throughout the necrosis and within the regions of (pyo) granulomatous inflammation (Fig 3A, B). Cells morphologically consistent with macrophages and neutrophils were positive for calprotectin, while epithelioid macrophages were negative. Necrotic regions showed variable amounts of positive staining which was not associated with intact cells or tissue; where appropriate, these areas were removed from image analysis when quantifying the total area of cell-specific calprotectin-positive immunolabelling. Differences in calprotectin expression did not infer infection between the pathogens in univariate analysis (P > 0.294).

## CD3 immunohistochemistry

Cells with moderate to strong membranous staining for CD3 (*i.e.* T-lymphocytes) were the second most common cell population

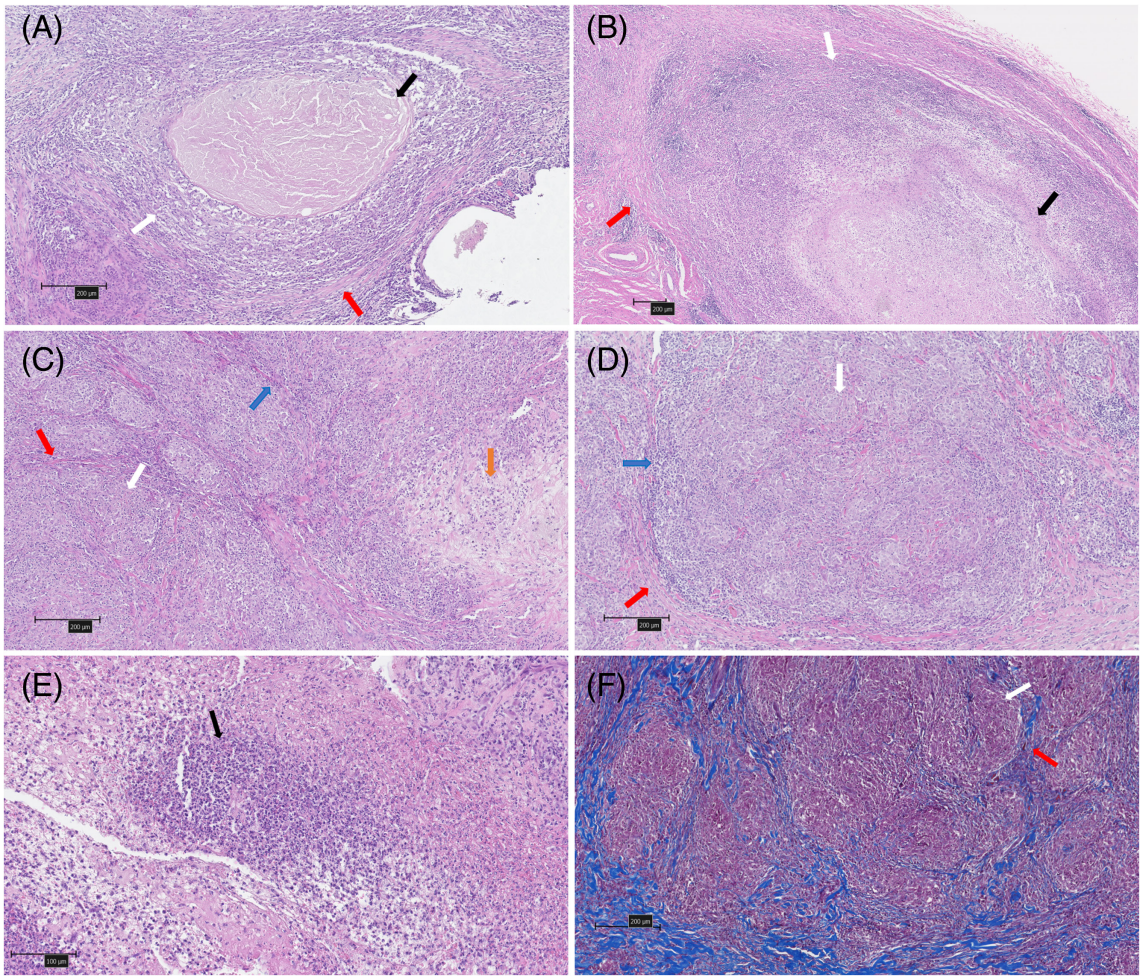


**FIG 1. Representative images of H&E (A-E) and Masson's Trichrome (F) stained feline tuberculosis lesions; "organised" granuloma, *M. bovis* (A, top left) with central necrosis (black arrow), macrophage and neutrophil cell layer (white arrow) and incomplete fibrous encapsulation (red arrow), magnification ×10, scale bar 200 μm; "organised" granuloma, *M. microti* (B, top right) showing a necrotic centre (black arrow) with a pyogranulomatous cellular layer (white arrow) and a thick fibrous capsule (red arrow), ×5, scale bar 200 μm; "atypical" granuloma, *M. bovis* (C, centre left) featuring small clusters of macrophages and some neutrophils (white arrows), with peripherally located lymphocytes (blue arrow) divided by fibrous septa (red arrows), with areas of degenerating collagen, oedema, and a mixed inflammatory cell population (orange arrow), magnification ×10, scale bar 200 μm; "atypical" granuloma, *M. microti* (D, middle right) displaying clusters of macrophages and neutrophils (white arrows), a peripheral zone of lymphocytes (blue arrow) and thin fibrous encapsulation (red arrow), ×10, scale bar 200 μm; "atypical" granuloma, *M. microti* (E, bottom left) cluster of necrotic neutrophils (black arrow), ×20, scale bar 100 μm; "atypical" granuloma, *M. microti* (F, bottom right) demonstrating the thin fibrous incomplete encapsulation (red arrow) around clusters of macrophages and neutrophils (white arrow), magnification ×10, scale bar 200 μm**

identified. They were located peripherally and scattered throughout all lesions (Fig 3C). The percentage-lesion positive staining for CD3 was not statistically significant different between the pathogens in univariate analysis ($P > 0.402$).

## Pax5 immunohistochemistry

Strong, diffuse nuclear staining for Pax5 was seen in cells towards the periphery of lesions. These B-cells were sometimes observed in clusters (Fig 3D). The total area of positive staining for Pax5 did not exceed 1% in any section, and there was comparatively greater staining of CD3 than Pax5 for each case (Table 2). Pax5 percentage-lesion positive staining did not differ between the pathogens, although the statistical significance when Pax5 positivity exceeded 0.50% was met for inclusion in multivariate analysis [P=0.186, OR=8.91 (0.35 to 228.43)], based on the *a priori* threshold for significance on univariate analysis of $P < 0.20$.

## Multivariate analysis

"Organised" vs "atypical" granulomas, amount of necrosis, BI category, Pax5 positive staining and gender were identified from univariate analysis for inclusion in a multivariate model. Age and tissue were also included as covariates. Preliminary exploration of the data showed that necrosis was highly collinear with granuloma type and was therefore excluded from multivariate modelling. The results of the final multivariate model identified no statistically significant factors suggesting infection with either *M. bovis* or *M. microti* (Table 4); granuloma type appeared to be the most important factor in this model [P=0.074, OR=0.01 (0.00005 to 1.52)], with *M. bovis* lesions being more likely to show "organised" granuloma morphology. The generalised variance inflation factor (GVIF) for all variables was calculated and normalised by the degrees of freedom; the corrected GVIF did not exceed 2.35 for any variable. A multidirectional stepwise algorithm was run

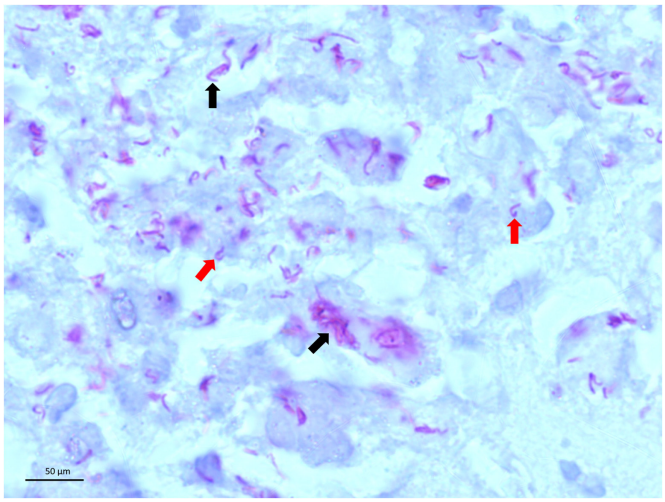


**FIG 2. Numerous intracellular acid-fast bacilli present in clusters (black arrows) or individually (red arrows) on Ziehl-Neelsen staining in a case of *Mycobacterium microti* infection (Case 12). This sample was scored as Bacterial Index 5. Note the characteristic S-shaped bacteria, ×1000. Scale bar 50 µm**

to identify the simplest model that could infer infection with either *M. bovis* or *M. microti*; this suggested a model with "organised" vs "atypical" granulomas as the only significant predictor for aetiology [P=0.016, OR=0.08 (0.01 to 0.63)].

## DISCUSSION

This study described and compared feline tuberculous lesions, using histology and IHC to identify potential features that could infer infection with either *M. bovis* or *M. microti* histologically at an early stage of the diagnostic investigation.

Among the companion animal population, the domestic cat appears to be much more susceptible to mycobacterial infections than other species, such as dogs (Broughan *et al.* 2013), and when outbreaks of disease do occur they can be devastating (O'Halloran *et al.* 2019). The role of companion animal species in the epidemiology of bovine TB in the UK is controversial; it has been shown that cats can become infected with the same *M. bovis* spoligotypes and genotypes as cattle (Monies *et al.* 2000, Gunn-Moore *et al.* 2011a), and endemic *M. microti* infection may provide a degree of protection against *M. bovis* infection (Smith *et al.* 2009). Others argue that cats most likely represent spill-over hosts for mycobacterial infections (Broughan *et al.* 2013). However, the high prevalence of TB in cats, particularly *M. bovis* infection (Gunn-Moore *et al.* 2011a), poses a real zoonotic risk to owners and the veterinary staff who handle and care for these animals (O'Connor *et al.* 2019). Both *M. bovis* and *M. microti* are capable of infecting cats and causing clinical disease. Differentiating between these two pathogens can be complicated, although the geographical location can suggest which is most likely (Gunn-Moore *et al.* 2011a). The reference standard diagnostic test is specialist mycobacterial culture, however, its sensitivity is only approximately 50%, and while more rapid diagnostic tests are available, they are non-validated and also have their limitations (Gunn-Moore 2014). The need for more commercially available and financially viable diagnostics for mycobacterial disease in cats is therefore imperative.

The median age of cats infected with *M. bovis* in the current study was higher than previously reported, whereas the age of *M. microti*-infected cats was similar (Gunn-Moore *et al.* 2011a). Neutered male cats with outdoor access were the largest demographic in the current study. Additionally, 19 of 22 cats were domestic short- or long-haired, consistent with previous findings (Gunn-Moore *et al.* 2011a). The single cat not reported to have outdoor access had been fed a commercially available raw food diet, that was previously implicated as the likely cause of an outbreak of *M. bovis* in predominantly indoor, pure-breed cats (O'Halloran *et al.* 2019).

The histopathological features of feline *M. bovis* and *M. microti* lesions has not been directly compared previously, and in this study, *M. bovis* lesions were more frequently classified as "organised" granulomas, with structural similarities to typical tuberculous granulomas observed in other species, *i.e.* a central necrotic core with a macrophage-dominant cellular layer and an outer rim of lymphocytes, with or without an external fibrous capsule (Pagán & Ramakrishnan 2018). In contrast with these descriptions, feline *M. bovis*-associated lesions often had abundant neutrophils present within zones of granulomatous inflammation, lacked MNGCs and did not show evidence of mineralisation. Conversely, lesions from cats infected with *M. microti* were more often composed of tightly packed, smaller macrophage clusters with or without neutrophils. These lacked a necrotic core in most cases, and were delimited by intermittent, thin capsules. To differentiate these from the previous presentation they were termed "atypical" granulomas.

These histopathological differences in the arrangement, organisation and structure of granulomas may be due to the host immune response to infection. Studies into the serum cytokine and chemokine profile of cats infected with mycobacteria show increased concentrations of tumour necrosis factor alpha (TNFα) in cats infected with *M. bovis* compared to those infected with *M. microti* (O'Halloran *et al.* 2018). TNFα is a potent pro-inflammatory cytokine, secreted primarily by monocytes, macrophages and dendritic cells (Van Crevel *et al.* 2002), and it plays an important role in the host immune response to mycobacterial infections through activating macrophages to promote phagocytosis and subsequent killing of mycobacteria, inducing expression of chemokines by macrophages resulting in the recruitment of $CD4^+$ T-lymphocytes, driving necrosis of infected macrophages, and the formation and maintenance of granulomas (Laster *et al.* 1988, Flynn *et al.* 1995, Roach *et al.* 2002, O'Garra *et al.* 2013, Dorhoi & Kaufmann 2014). It also has been shown to stimulate fibroblasts (Battegay *et al.* 1995), which may contribute to the deposition of collagen fibres to encapsulate granulomas. However, excessive TNFα may result in disruption of normal granuloma formation, with overactivation of macrophages resulting in increased necrosis with release of intracellular mycobacteria into the necrotic milieu (Dorhoi & Kaufmann 2014). Conversely, cats with *M. microti* showed increased levels of platelet-derived growth factor-BB (PDGF-BB), which is a potent mitogen of

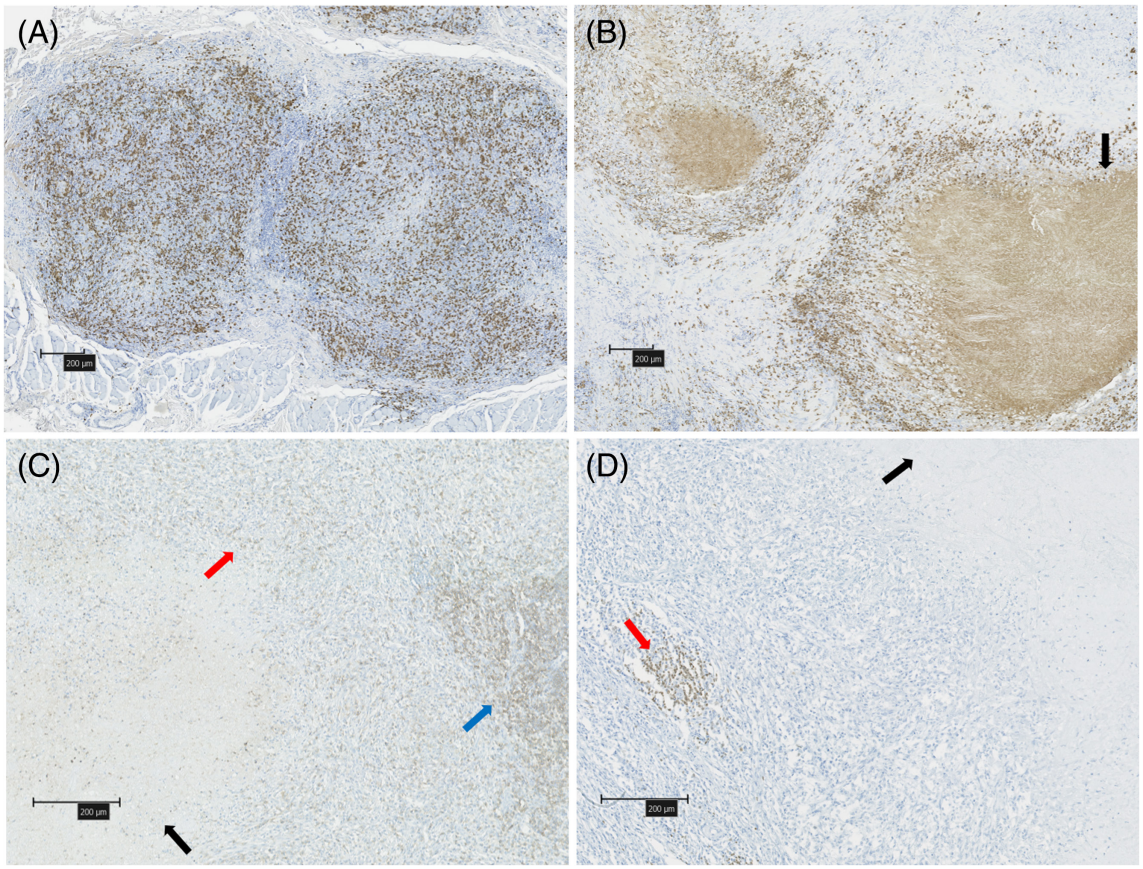


**FIG 3. Immunohistochemistry of feline tuberculosis lesions to assess (A) calprotectin expression, ×5; (B) calprotectin expression in regions of necrosis (black arrow), ×5; (C) CD3 expression in a tuberculous lymph node lesion (red arrow) and effaced remnants of paracortical lymph node tissue (blue arrow), with minimal CD3-positive cells in regions of necrosis (black arrow) ×10; (D) expression of Pax5 in a subcutaneous tuberculous lesion (red arrow), with no positive staining cells in necrotic regions (black arrow), ×10. Scale bar 200 μm**

**Table 4. Results of multivariate logistic regression, modelling factors where P <0.20 on univariate analysis**

| Variable | Summary statistics [median (range)] | | Odds ratio | 95% CI | P-value |
|---|---|---|---|---|---|
| | *M. bovis* | *M. microti* | | | |
| Overall | — | — | — | — | 0.113 |
| Structure | A: 3, O: 8 | A: 9, O: 2 | 0.08 | 0.00005 to 1.52 | 0.074 |
| Pax5 | 0.16% (<0.01 to 0.83%) | 0.07% (<0.01 to 0.72%) | | | |
| 0.00 to 0.09% | 4 | 6 | Baseline | — | — |
| 0.10 to 0.50% | 4 | 3 | 18.94 | 0.46 to 788.00 | 0.122 |
| >0.50% | 3 | 2 | 0.02 | 0.00001 to 42.10 | 0.323 |
| Gender | MN: 5, FN: 6 | MN: 8, FN: 3 | 0.08 | 0.003 to 2.30 | 0.144 |
| BI category | L: 4, H: 7 | L: 8, H: 3 | 0.02 | 0.00005 to 4.56 | 0.150 |
| Age | 5y 1m (1y 0m to 12y 1m) | 7y 0m (1y 5m to 15y 0m) | 1.02 | 0.97 to 1.09 | 0.364 |
| Tissue | Skin: 6, LN: 5 | Skin: 8, LN: 3 | 0.31 | 0.01 to 9.31 | 0.507 |

BI Bacterial Index, A "Atypical", O "Organised", MN Male neutered, FN Female neutered, L Low, H High, y Years, m Months, LN Llymph node, CI Confidence interval
The data are adequately described by the model (Hosmer-Lemeshow goodness of fit, P=0.235)

fibroblasts and plays an important role in fibroblast proliferation and deposition of collagen (Agren *et al.* 1999, Van Der Kroef *et al.* 2020). It has also been shown that PDGF-BB enhances TNFα-induced chemokine secretion by fibroblasts and production of collagen (Van Der Kroef *et al.* 2020). The increased levels of TNFα in cats with *M. bovis*-infection may contribute to more necrosis within lesions, while increased PDGF-BB in *M. microti*-infected cats may result in differences in the pattern of collagen deposition, resulting in smaller granulomas divided by fibrous septa. Differences in the chronicity of the lesion may also play a role, with increased deposition of collagen as lesions progress and become more chronic as reported for cattle (Wangoo *et al.* 2005). However, due to the clinical nature of the submitted samples it is unknown how long the lesions had been present or when infection occurred. It is therefore not possible to classify samples according to age or know how long the lesions had to develop the structural organisation of the (pyo)granulomatous infiltrate observed. It is possible that the variability in the presentation

stems from the bacteria themselves, and one can hypothesise that these histological differences could be mediated by key virulence factors. It has been shown that ESAT-6 is a leucocidin resulting in neutrophil necrosis (Francis *et al*. 2014); this virulence factor may contribute to the increased amount of necrosis in *M. bovis*-associated lesions. Furthermore, granuloma type and macrophage aggregation is disrupted following infection with RD1 mutant mycobacteria in an experimental setting (Volkman *et al*. 2004, Davis & Ramakrishnan 2009); the lesions described in this study from cats with *M. microti* infection, which can be thought of as a natural RD1 deletion mutant (Frota *et al*. 2004), would appear to support this finding that RD1 proteins play a role in the structure and organisation of mycobacterial granulomas.

AFB were present in 91% of the lesions examined in the current study, in contrast with previous studies which reported that ZN-positive organisms morphologically consistent with mycobacteria were identified in only approximately 30% (37/134) of feline mycobacterial histopathological samples (Gunn-Moore *et al*. 2013). As has been previously reported, *M. microti* AFB often presented with an S-shaped morphology (Van Soolingen *et al*. 1998). It is of note that identification of AFB can be analyst dependent (Gunn-Moore *et al*. 2011b), and mycobacteria can alter their cell wall composition, resulting in a loss of "acid-fastness" on ZN staining (Seiler *et al*. 2003). Application of the BI scoring system when reporting on ZN-staining of diagnostic samples is recommended for future studies, as it enables more consistent reporting compared to subjective terms to describe the number of ZN-positive organisms present. For example, this is likely to provide a robust reference in the comparative assessment of diagnostic tests, *e.g*. the likelihood of obtaining a positive PCR result on FFPE tissues with differing BI scores. Of interest, 45% (10/22) of samples had a high BI in this study, and one *M. microti* sample even scored as grade 5 (100.01 to 1000 AFB/hpf). This finding is directly relevant for diagnostics, as feline TB is often thought of as a paucibacillary infection, while multibacillary lesions typically indicate infection with non-tuberculous, and therefore non-zoonotic, mycobacterial species (Davies *et al*. 2006). The data presented here show that feline MTBC infections can present with abundant AFB, so discriminating between TB and NTM infections based on the number of AFB and BI scoring is not possible.

This study did not identify differences between the area of calprotectin-positivity in *M. bovis* and *M. microti* lesions. Calprotectin is expressed by circulating monocytes and those freshly recruited to developing areas of (pyo)granulomatous inflammation, plus granulocytes and some subsets of activated macrophages (Zwadlo *et al*. 1986). As monocytes differentiate into macrophages there is a loss of calprotectin expression, whereas expression of CD163 is upregulated (Zwadlo *et al*. 1985). The high proportion of lesions expressing calprotectin suggests active development of the inflammatory infiltrate, with ongoing recruitment of monocytes and neutrophils (Rugtveit *et al*. 1996). Many macrophages, notably those with an epithelioid phenotype, did not express this molecule, suggesting differentiation and subsequent loss of calprotectin expression; this is consistent with what has been previously reported in cases of feline mycobacteriosis (Kipar *et al*. 2003). The proportion of calprotectin-positive cells has been shown to decrease in more chronic tuberculous granulomas in other species (García-Jiménez *et al*. 2012, García-Jiménez *et al*. 2013), therefore the tissues in this study with lower levels of calprotectin expression could reflect the presence of more chronic lesions. While identification of calprotectin-positive cells can give an insight to the active recruitment of monocytes and granulocytes to the granuloma, it does not fully characterise the macrophage cell populations present, which is a limitation of this study. Alternative pan-macrophage markers, such as ionised calcium-binding adapter molecule 1 (Iba1), may be more specific for detection of macrophage populations within tuberculous lesions (Pierezan *et al*. 2014), while staining for CD163 could identify anti-inflammatory M2 macrophage populations (McBride *et al*. 2017). Additionally, markers such as Ki-67 could be used to identify local macrophage proliferation (McBride *et al*. 2017), providing further description of these lesions above what can be achieved by staining for calprotectin alone.

No differences were observed between *M. bovis* and *M. microti* lesions regarding positivity for T- or B-lymphocyte markers. Both T- and B-lymphocytes were located peripherally in tuberculous lesions in the current study and were a minor component of the inflammatory infiltrate. For each cat, the area of positive staining for T-lymphocytes exceeded that for B-lymphocytes, suggesting T-lymphocytes may play a greater role to the composition of the granuloma and to the pathogenesis of feline MTBC infections. In other species, T-lymphocytes expressing CD3 appear to be present within tuberculous lesions at consistent levels across different stages of granuloma development (García-Jiménez *et al*. 2013, Salguero *et al*. 2017). The role of T-lymphocytes in the pathogenesis of tuberculous infections, characteristic of a type IV hypersensitivity response, has been well studied and reviewed (Schluger & Rom 1998, O'Garra *et al*. 2013); $CD4^+$ lymphocytes, and in particular T-helper 1 cells, play an important role in developing protective immunity against mycobacteria as well as stimulating macrophage activity via secretion of IFN$\gamma$. $CD4^+$-deficient individuals have been shown to be more susceptible to mycobacterial infections (Geldmacher *et al*. 2008), and idiopathic $CD4^+$ lymphocytopenia has been documented in a cat with disseminated *M. xenopi* infection (Meeks *et al*. 2008). The role of other T-cell subsets, including CD8+ and $\gamma\delta$ TCR+ T-cells, are less well elucidated (Schluger & Rom 1998), although it has been suggested that $\gamma\delta$ T-cells may play an important role in the pathogenesis of bovine mycobacterial infections (Guzman *et al*. 2012). The proportion of B-lymphocytes expressing CD20 or CD79a, appears to increase in some species (García-Jiménez *et al*. 2012, Vallejo *et al*. 2018), possibly indicating a shift towards humoral immunity as the disease progresses. The role of B-lymphocytes in the pathogenesis of TB has been often overlooked, although there is gathering evidence that B-lymphocytes may interact with and regulate the response of T-lymphocytes in chronic infections, in addition to driving differentiation of alternatively-activated M2 macrophages, which play a modulatory role in mycobacterial infections (Martinez & Gordon 2014). Plasma cells, which were rarely identified on H&E-stained sections, do not express Pax5 (Barberis *et al*. 1990, Feldman & Dogan 2007), so they were not

identified by IHC in this study; their contribution to tuberculous lesions in cats could be explored further through use of markers such as immunoglobulin λ light-chain (Mellor *et al*. 2008). However, the results of the current study appear consistent with findings observed in tuberculous lesions in other species (Kipar *et al*. 2003, García-Jiménez *et al*. 2013).

Tuberculosis affects a wide range of organs and body systems, but histological and immunological studies have normally been performed on lung or lymph node granulomas (Turner *et al*. 2003, Wangoo *et al*. 2005, García-Jiménez *et al*. 2012, García-Jiménez *et al*. 2013); since feline tuberculous lesions are predominantly cutaneous, this may explain why previously established granuloma scoring systems did not correspond well to feline lesions. A review of cutaneous mycobacterial lesions in humans identified seven different histopathological patterns, including "organised" tuberculoid granulomas, but it was also noted that multiple patterns could be present within the same biopsy sample examined and that the histopathological presentation of disease could not classify infection with different mycobacterial species (Santa Cruz & Strayer 1982). Neutrophils also appeared to be more frequently identified in cutaneous mycobacterial lesions (Santa Cruz & Strayer 1982). Differences in host-pathogen interactions between host species have also been identified; human *M. tuberculosis* infection typically results in the formation of caseous granulomas, whereas non-necrotic lesions are more common in some strains of mice (Kramnik *et al*. 2000). Similarly, *M. microti* infection in its maintenance host, the field vole, often results in caseous necrosis with associated regions of dystrophic mineralisation, and MNGCs (Kipar *et al*. 2014), features which were mostly absent from feline lesions. Studies into the histological and immunological appearance of mycobacterial lesions affecting other organs, such as the eye, could provide further insights as to whether lesion development varies due to the tissue or organ infected, the infecting organisms present, or whether the feline immune response differs compared to that in other species. As this study used clinical cases of naturally occurring TB, managed by referring veterinarians, data such as the chronicity of lesions and the duration of formalin-fixation of the sample was unavailable, so it is not possible to exclude that some of the histological and immunological differences could be explained by this.

The results of the histological analyses warrant further investigation on a larger set of confirmed tuberculous samples as the small sample size may be insufficient to support a multivariate model. Furthermore, categorising the continuous variables that did not meet the assumptions for binomial logistic regression results in a loss of statistical power and the interpretation of any statistically significant findings is more complicated. Additionally, multicollinearity of some of the variables in this study was identified; where appropriate, collinear variables were excluded from models which were deemed to not result in a loss of biological explanation of the results. Despite the above limitations, in this study the granuloma type was the most statistically significant finding in suggesting whether a cat was infected with *M. bovis* or *M. microti*. This is relevant, as the findings of this study are useful in the diagnostic histopathology setting to infer the likelihood of infection with either *M. bovis* or *M. microti* before further testing, *i.e*. culture, PCR or IGRA.

In conclusion, this study characterised the histological and immunohistochemical appearance of tuberculous lesions in cats naturally infected with *M. bovis* or *M. microti*. With ZN staining, *M. microti* AFB were frequently S-shaped, and numerous AFB were observed in individual cases for both *M. bovis* and *M. microti* infections. Therefore, abundant ZN-positive organisms are not a specific diagnostic feature of feline infection with non-zoonotic NTM species. The study identified differences in the histopathological features between these two mycobacterial species that may raise the index of suspicion for infection with either mycobacterial species at an earlier stage of the clinical investigation of these cases.

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## Conflict of interest

None of the authors of this article has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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## Supporting Information

The following supporting information is available for this article:

**Appendix S1.** Macro scripts used for quantifying positive staining for collagen (Masson's Trichrome) and immunohistochemical markers.

## 3.2 Supplementary Material

Calculation of the area of positive staining for collagen on MT-stained slides, and for positive immunohistochemical labelling with calprotectin, CD3 and Pax5 respectively, was achieved using a macro script written in FIJI (Schindelin *et al.*, 2012). These scripts were uploaded as supplementary material to the published paper and are presented herein.

### 3.2.1 Masson's Trichrome Positive Staining and Total Area Macro

```
rename("Image");

run("Set Scale...", "distance=0.3390 known=1 pixel=1 unit=µm global");

run("Colour Deconvolution", "vectors=[Alcian blue & H]");

close("Colour Deconvolution");

close("Image-(Colour_2)");

close("Image-(Colour_3)");

selectWindow("Image-(Colour_1)");

setThreshold(0, 160);

//setThreshold(0, 160);

setOption("BlackBackground", false);

run("Convert to Mask");

run("Set Measurements...", "area integrated limit redirect=None decimal=3");

run("Measure");

selectWindow("Image");

run("8-bit");

setThreshold(0, 254);
```

```
//setThreshold(0, 254);

setOption ("BlackBackground", false);

run("Convert to Mask");

run("Set Measurements...", "area integrated limit redirect=None decimal=3");

run("Measure");
```

## 3.2.2 Calprotectin

### 3.2.2.1 Calprotectin Positive Staining Macro

```
rename("Image");

run("Set Scale...", "distance=0.4006 known=1 pixel=1 unit=µm global");

run("Colour Deconvolution", "vectors=[H DAB]");

close("Colour Deconvolution");

close("Image-(Colour_1)");

close("Image-(Colour_3)");

selectWindow("Image-(Colour_2)");

setThreshold(0, 211);

//setThreshold(0, 211);

setOption("BlackBackground", false);

run("Convert to Mask");

run("Set Measurements...", "area integrated limit redirect=None decimal=3");

run("Measure");
```

### 3.2.2.2 Calprotectin Total Lesion Area Macro

```
rename("Image");

run("Set Scale...", "distance=0.4006 known=1 pixel=1 unit=µm global");

selectWindow("Image");

run("8-bit");

setThreshold(0, 254);

//setThreshold(0, 254);

setOption ("BlackBackground", false);

run("Convert to Mask");

run("Set Measurements...", "area integrated limit redirect=None decimal=3");

run("Measure");
```

## 3.2.3 CD3 Positive Staining and Total Lesion Area Macro

```
rename("Image");

run("Set Scale...", "distance=0.3148 known=1 pixel=1 unit=µm global");

run("Colour Deconvolution", "vectors=[H DAB]");

close("Colour Deconvolution");

close("Image-(Colour_1)");

close("Image-(Colour_3)");

selectWindow("Image-(Colour_2)");

setThreshold(0, 207);

//setThreshold(0, 207);
```

```
setOption("BlackBackground", false);

run("Convert to Mask");

run("Set Measurements...", "area integrated limit redirect=None decimal=3");

run("Measure");

selectWindow("Image");

run("8-bit");

setThreshold(0, 254);

//setThreshold(0, 254);

setOption ("BlackBackground", false);

run("Convert to Mask");

run("Set Measurements...", "area integrated limit redirect=None decimal=3");

run("Measure");
```

## 3.2.4 Pax5 Positive Staining and Total Lesion Area Macro

```
rename("Image");

run("Set Scale...", "distance=0.2754 known=1 pixel=1 unit=µm global");

run("Colour Deconvolution", "vectors=[H DAB]");

close("Colour Deconvolution");

close("Image-(Colour_1)");

close("Image-(Colour_3)");

selectWindow("Image-(Colour_2)");

setThreshold(0, 213);
```

```
//setThreshold(0, 213);

setOption("BlackBackground", false);

run("Convert to Mask");

run("Set Measurements...", "area integrated limit redirect=None decimal=3");

run("Measure");

selectWindow("Image");

run("8-bit");

setThreshold(0, 254);

//setThreshold(0, 254);

setOption ("BlackBackground", false);

run("Convert to Mask");

run("Set Measurements...", "area integrated limit redirect=None decimal=3");

run("Measure");
```

## 3.3. Discussion

In this study a novel, descriptive-based scoring metric was established to describe the histopathological features of feline (muco)cutaneous and lymph node tuberculous lesions in cats. It was shown that cases of *M. bovis* were associated with 'organised' granulomas, characterised by central caseous necrosis surrounded by macrophages, epithelioid macrophages and neutrophils, fewer circumferential and interspersed lymphocytes, and variable encapsulation. These were similar in appearance to typical tuberculous granulomas (Pagán and Ramakrishnan, 2018), although features such as mineralisation and the presence of MNGCs were lacking. In contrast, *M. microti*-associated lesions showed a different histopathological appearance of well-defined small clusters of macrophages and epithelioid macrophages with occasional neutrophils and fewer lymphocytes, often surrounded by thin fibrous septa and lacking central necrosis; these were termed 'atypical' granulomas. Identification of these features could infer infection with either *M. bovis* or *M. microti* at an earlier stage in the diagnostic investigation, allowing for more rapid implementation of safety measures to protect and safeguard human health. This study also implemented a grading system used for cases of human leprosy to allow for more objective reporting of the number of AFB present within feline mycobacterial lesions and showed that cases of TB can present with large numbers of AFB, a feature often attributed to infection with NTM (Malik *et al.*, 2002, O'Brien *et al.*, 2017a), especially non-zoonotic species (O'Brien *et al.*, 2017c). While no differences were identified between *M. bovis* and *M. microti* lesions based on immunohistochemical staining, this study created a set of macros to automate the analysis of calculating the area of the lesion staining positive for these cell type specific markers which could be adapted for other research studies, or validated for diagnostic purposes (Mulrane *et al.*, 2008).

Although the number of cases in this study is small, it provides a foundation for further histopathological studies into feline mycobacterial lesions. The histopathological features of feline TB lesions differ from those seen in cattle (Wangoo *et al.*, 2005), hence the development of a description-based scoring system specific for cats. This is in part likely due to TB in cats resulting from infection with either *M. bovis* or *M. microti*, which this study shows can result in different histopathological features. This could be due to the absence of RD1 encoded virulence factors in *M. microti* (Orgeur *et al.*, 2021). However, there are other features associated with TB in other species that appear to be rare in cases of feline TB regardless of the underlying aetiology, namely mineralisation and MNGCs (Kipar *et al.*, 2003, Gunn-Moore *et al.*, 2011b). Classification systems for feline mycobacterial lesions are not without controversy; the terms tuberculoid leprosy and lepromatous leprosy have been applied for cases of FLS (Malik *et al.*, 2002), although it has been disputed whether these terms are appropriate given the lack of differential cytokine expression between 'tuberculoid' and 'lepromatous' lesions in cats (Kipar *et al.*, 2003), compared to what is seen in humans (Yamamura *et al.*, 1991). Therefore, this novel descriptive classification should be applied to a larger set of tissues from cases of feline cutaneous and lymph node TB, as well as lesions from other tissue types such as the eye (Chapter 2), and then applied to a set of tissue samples without aetiological confirmation to provide external validation of this proposed classification. It should also be investigated how this classification applies to non-tuberculous feline

lesions. With a few exceptions, NTM are RD1 negative (van Ingen *et al.*, 2009), like *M. microti*, therefore there may be greater similarity between *M. microti* and NTM lesions than between feline MTBC infections. Similarly, the adapted ZN-scoring system should also be applied on further tissue samples with and without an aetiological diagnosis to provide further information as to whether the number of AFB can be used to either differentiate between *M. bovis* and *M. microti*, as has been suggested in some studies (Gunn-Moore *et al.*, 2011b), or to identify the proportion of cases of TB scoring with a high BI which could be erroneously diagnosed as FLS (O'Halloran *et al.*, 2016). It could also be beneficial to have a BI score or grade when attempting to develop molecular based diagnostic tests for use on FFPE tissues (Chapter 7), to determine whether successful DNA extraction and obtaining a positive PCR result is dependent on the bacterial burden in the sample that can be visualised with ZN staining.

While no differences were identified between *M. bovis* or *M. microti* lesions on IHC, the findings of this study were broadly similar to other immunohistochemical studies on feline cutaneous mycobacterial lesions, with a dominance of myeloid-derived cells that were positive for calprotectin, fewer T-cells and lesser B-cells (Kipar *et al.*, 2003). The panel of immunohistochemical markers in this study was small and could be expanded upon to further characterise the cell populations present within TB lesions. This could include T-cell subset markers such as CD4 and CD8 (Turner *et al.*, 2003), as this may provide further insights into the development and turnover of granulomas in cases of TB (Palmer *et al.*, 2007). Cytokine markers could also be investigated such as TNF-α, given this was increased in cats with *M. bovis* infection compared to those with *M. microti* (O'Halloran *et al.*, 2018b). If IHC findings corroborate the serum cytokine analysis, this would strengthen the evidence of differential immune responses in the face of infection with different members of the MTBC; however, other factors such as the duration of infection, the infective dose, and the route of infection likely play an important role in the host immune response generated. It would also expand our understanding of the feline immune response to *M. microti* infection and how this differs to infection of this pathogen in its maintenance host, (*i.e.,* the vole) where granulomas with central caseous necrosis are identified (Kipar *et al.*, 2014).

To summarise, this study demonstrated that the histopathological features of feline TB infections differ to the extent that the type of granuloma present differs between infection with either *M. bovis* (‘organised’) or *M. microti* (‘atypical’). This study also provides the largest singular description of the histopathological and immunohistochemical features of feline *M. microti* cutaneous and lymph node lesions, expanding our knowledge of this pathogen within a host where it can cause significant disease. It was also shown that both *M. bovis* and *M. microti* can present with large numbers of AFB on ZN staining, providing further evidence that cases of mycobacteriosis with abundant AFB cannot be assumed to be non-tuberculous or non-zoonotic. Therefore, histopathology may provide an early means of suspecting infection with *M. bovis*, which may be useful in edge areas of GB where both *M. bovis* and *M. microti* are prevalent (Gunn-Moore *et al.*, 2011a, More *et al.*, 2018), prior to performing tests of specific pathogen detection such as culture and/or PCR, or immunological testing such as the IGRA.

# Chapter 4: IGRA Testing in Cases of Feline Mycobacteriosis: Diagnostic Accuracy and Serial Testing to Monitor Treatment Responses

## Preface

The following chapter consists of two articles, published in Preventive Veterinary Medicine, and Pathogens, respectively. Crown Copyright is asserted for the article published in Preventive Veterinary Medicine. Author Rights are granted to use the Preprint, Accepted Manuscript and Published Journal Article for Personal Use, Internal Institutional Use and Scholarly Sharing Purposes, including submission in a non-commercially published thesis. The Authors retain copyright for the article published in Pathogens, which is available as an Open Access publication.

**Mitchell, J. L.**, Stanley, P., McDonald, K., Burr, P., Rhodes, S. G., Gunn-Moore, D. A. and Hope, J. C. Diagnostic accuracy of the interferon-gamma release assay (IGRA) for cases of feline mycobacteriosis. *Preventive Veterinary Medicine*. 2021; 193, article number 105409.

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## Author's Contribution

The author was responsible for collating and interpreting all clinical case data, performing the statistical analysis, and writing the manuscript for both published articles.

## 4.1 Introduction

While histopathology plays an important role in the initial diagnosis of mycobacterial infections, and the previous chapter shows there may be some features which could help differentiate between the two causative agents of TB in cats, further diagnostics are required to provide a group or species-level diagnosis of mycobacteriosis. Indirect tests, such as immunological assays, provide one means of achieving this. Tests of CMI for the diagnosis of mycobacterial infections are commonplace in humans (Mack *et al.*, 2009, Idh *et al.*, 2010, Muñoz *et al.*, 2015) and cattle (de La Rua-Domenech *et al.*, 2006, Bezos *et al.*, 2014), yet their utility in cats has been limited. Tuberculin skin testing in cats is considered unreliable and is not recommended (Cousins and Florisson, 2005); however, the IGRA has been successfully adapted for use in this species (Rhodes *et al.*, 2008b), and has been commercially available as a diagnostic test for feline mycobacteriosis since 2013.

Although the feline IGRA has been in use for nearly 10 years, there is very limited data on its performance characteristics, as well as the range of applications for which it can be applied. Previous studies have shown excellent sensitivity for MTBC infections with reported good diagnostic power to differentiate between *M. bovis* and *M. microti* infections, whereas comparatively the sensitivity of the IGRA for NTM infections is poor (Rhodes *et al.*, 2011). There are also concerns surrounding test interpretation, for both MTBC and NTM infections (O'Halloran and Gunn-Moore, 2017, Černá *et al.*, 2020). It is reported that up to 20% of cats infected with *M. bovis* do not generate a positive response to the antigenic cocktail of ESAT-6/CFP-10 (Rhodes *et al.*, 2011); therefore, these cats may have a PPDB-biased, ESAT-6/CFP-10 negative response, and be misinterpreted as infection with *M. microti*. If such a result is seen in a cat from areas of GB that are high risk for bTB *i.e.*, south west England (More *et al.*, 2018), a clinically informed judgement can be made that this is likely to be a case of *M. bovis* infection that is non-responsive to ESAT-6/CFP-10. This can be done as feline *M. bovis* infections co-localise with the distribution of *M. bovis*-positive cattle herds (Gunn-Moore *et al.*, 2011a). However, if a PPDB-biased, ESAT-6/CFP-10 negative IGRA result is seen in a cat from 'edge areas', this result cannot be definitively attributed to *M. microti* infection, as it may be a *M. bovis* infection without an ESAT-6/CFP-10 specific immune response. This may have potential implications if there are immunocompromised individuals in the household, as they may be unknowingly putting themselves at higher risk of becoming infected if they are dealing with a *M. bovis*-infected cat when it has been assumed that infection is due to *M. microti* (Human Animal Infections and Risk Surveillance (HAIRS) group, 2014, O'Connor *et al.*, 2019). Regarding NTM infections, a PPDA-biased response is routinely reported as inferring infection with *M. avium* (Gunn-Moore, 2014), while a negative IGRA in the face of ZN-positive organisms infers infection with a non-*M. avium* NTM (O'Halloran and Gunn-Moore, 2017). However, it has been shown that some non-*M. avium* NTM species can induce a PPDA-biased response (Rhodes *et al.*, 2011, Černá *et al.*, 2020), potentially resulting in a misdiagnosis and implementation of a suboptimal treatment protocol. More concerningly, non-*M. avium* NTM species may also be misdiagnosed as cases of MTBC infection (Černá *et al.*, 2020), which may result in potentially unnecessary treatment with antimicrobials that need to be safeguarded for human health (World Health

Organization, 2019a). Previous studies on the performance of the feline IGRA have been on small populations of cats, and the methodology of test result interpretation has changed from readouts of IFNγ concentrations and determining antigen positivity based on individual results (Rhodes *et al.*, 2008a, Rhodes *et al.*, 2011) to the establishment of defined cut-off optical density (OD) values (Mitchell *et al.*, 2021e). Therefore, it is critical to re-evaluate the performance of the IGRA now that it is routinely used as a diagnostic test for cases of feline mycobacteriosis.

While the scope of the feline IGRA has been for the diagnosis of mycobacterial infections in cats with clinical signs suggestive of mycobacterial disease, use of the test for the monitoring of treatment responses has also been suggested (Rhodes *et al.*, 2011). There is a lack of understanding of the feline immune response to mycobacterial infections which has precluded the routine use of serial IGRA testing for monitoring treatment responses. For example, should we aim for reversion to negativity before stopping treatment, suggesting that the antigenic stimulus driving the T-cell response detected by the IGRA has been eliminated (Pathan *et al.*, 2001, Kaech *et al.*, 2002), or does remaining IGRA positive demonstrate the retention of a memory T-cell response which can provide longer-lasting protective immunity (Bosshard *et al.*, 2009, Bugiani *et al.*, 2011)? Answering this question would be of clinical benefit as attaining a negative end of treatment IGRA result may provide a metric by which the decision to stop treatment can be made.

The aims of this chapter, presented in the following two papers, were twofold. Firstly, to thoroughly evaluate the test performance characteristics *i.e.*, sensitivity, specificity, and likelihood ratios (LR) of the feline IGRA compared to the results of mycobacterial culture and/or PCR, as well as the agreement between test results, based on current test interpretation. Receiver-operator characteristic (ROC) curve analysis was undertaken to explore new cut-off values to define antigen positivity; these suggestive values were then applied to the set of test results for re-interpretation and reassessment of test sensitivity, specificity, LR, and agreement. Evaluation and re-assessment of positive and negative control thresholds was also undertaken, with subsequent analysis to try and identify factors associated with positive control test failure. Secondly, the results of cats that had undergone serial IGRA testing over the course of treatment for mycobacteriosis were investigated to describe the pattern of responses seen, as well as exploring whether remaining IGRA positive at the end of treatment was associated with recurrence of disease, among other factors. The results of this chapter could provide further data for the validation of the IGRA as an approved diagnostic test for mycobacterial infections in cats (Middlemiss and Clark, 2018), and to help answer key clinical questions regarding the utility of serial IGRA testing for monitoring treatment responses and the significance of the end of treatment test result on longer-term outcomes.

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# Diagnostic accuracy of the interferon-gamma release assay (IGRA) for cases of feline mycobacteriosis

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ABSTRACT

The aim of this study was to evaluate the sensitivity and specificity of the interferon-gamma release assay (IGRA) for diagnosing infections with members of the *Mycobacterium* (*M.*) *tuberculosis*-complex (MTBC) and non-tuberculous mycobacteria (NTM) in domestic cats, and to generate defined feline-specific cut-off values using receiver operating characteristic (ROC) curve analysis to improve test performance.

Records of 594 cats that had been tested by IGRA were explored to identify individuals that had a culture and/or polymerase chain reaction (PCR)-confirmed case of mycobacterial disease, and those that had a final diagnosis of non-mycobacterial disease. A total of 117 cats - 80 with mycobacterial disease and 37 diagnosed with a condition other than mycobacteriosis - were identified for further detailed analysis. This population was used to estimate test sensitivity and specificity, as well as likelihood ratios for the IGRA to correctly identify a cat with or without mycobacterial disease. Agreement between IGRA results and culture/PCR using current and proposed new cut-off values was also determined.

ROC analysis of defined confirmed infected and non-mycobacterial disease control cats allowed an adjustment of current test cut-offs that increased the overall test sensitivity for MTBC infections from 83.1 % (95 % confidence interval [CI]: 71.5–90.5 %) to 90.2 % (95 % CI: 80.2−95.4%), and *M. bovis* infection from 43 % (95 % CI: 28.2−60.7%) to 68 % (95 % CI: 51.4−82.1%) while maintaining high test specificity (100 % in both cases). Overall agreement between IGRA results and culture/PCR, while recognising that neither culture nor PCR tests have perfect sensitivity, improved from weak ($\kappa = 0.57$) to moderate ($\kappa = 0.71$) using new proposed IGRA test cut-off values.

Application of these results, based upon the statistical analysis of accumulated test data, can improve the diagnostic performance of the feline IGRA, particularly for identifying infections with *M. bovis*, without compromising specificity.

*Abbreviations:* AUC, area under the curve; BCG, Bacillus Calmette-Guérin; CD, cluster of differentiation; CI, confidence interval; CV, coefficient of variation; DPBS, Dulbecco's Phosphate-Buffered Saline; DxH, domestic short/medium/longhair cat; ELISA, enzyme-linked immunosorbent assay; ESAT-6/CFP-10, 6kDa early secreted antigenic target and 10kDa culture filtrate protein; FeLV, feline leukaemia virus; FIV, feline immunodeficiency virus; IFNγ, Interferon-gamma; IGRA, interferon-gamma release assay; IL, interleukin; LR, likelihood ratio; *M.*, *Mycobacterium*; MTBC, *Mycobacterium tuberculosis*-complex; NC, negative control; NK, Natural Killer; NPV, negative predictive value; NTM, non-tuberculous mycobacteria; OD, optical density; OR, odds ratio; PBMCs, peripheral blood mononuclear cells; PC, positive control; PCR, polymerase chain reaction; PMA-Ca, phorbol myristate acetate-calcium ionophore; PPD, purified protein derivative; PPDA, purified protein derivative from *Mycobacterium avium*; PPDB, purified protein derivative from *Mycobacterium bovis*; PPV, positive predictive value; QC, quality control; RD-1, region of difference-1; ROC, receiver operator characteristic; SD, standard deviation; TB, tuberculosis; ZN, Ziehl-Neelsen.

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## 1. Introduction

The interferon-gamma (IFNγ) release assay (IGRA) is an *in vitro* test initially designed for identifying cattle infected with *Mycobacterium* (*M.*) *bovis* in Australia as part of the national bovine tuberculosis (TB) eradication campaign (Wood et al., 1990). It has since been adapted for the diagnosis of TB in humans (Streeton et al., 1998), deer (Slobbe et al., 2000; Waters et al., 2008), and alpacas (Rhodes et al., 2012). The IGRA has also been modified to diagnose cases of tuberculous and non-tuberculous mycobacterial disease in the domestic cat (Rhodes et al., 2008b), and has been commercially available through Biobest Laboratories, Edinburgh, Scotland, since 2013.

The IGRA assesses the cell-mediated immune response to mycobacterial infections. In domestic cats this involves the incubation of isolated peripheral blood mononuclear cells (PBMCs) with a panel of mycobacterial antigens consisting of avian tuberculin (purified protein derivative [PPD] A), bovine tuberculin (PPDB) and an antigenic peptide cocktail of the region of difference-1 (RD-1) proteins 6 kDa early secreted antigenic target and 10 kDa culture filtrate protein (ESAT-6/CFP-10). Antigen-specific T-lymphocytes from cats infected with mycobacteria secrete IFNγ into the supernatant upon incubation with mycobacterial antigens, which is then detected by an enzyme-linked immunosorbent assay (ELISA) and the results reported as optical density (OD) values (Rhodes et al., 2011; O'Halloran et al., 2019). Isolated PBMCs from cats that have not been infected or exposed to mycobacteria should not secrete significant levels of IFNγ in response to incubation with mycobacterial antigens (Rhodes et al., 2008a, 2011). The responses to PPDA, PPDB and ESAT-6/CFP-10 are then used to interpret the likelihood of *M. tuberculosis*-complex (MTBC) infection (where the PPDB response exceeds the PPDA response) and *M. bovis* in particular (where in addition to the above a response to ESAT-6/CFP10 is also observed) (Rhodes et al., 2008b).

The development of a feline-specific IGRA has been greatly beneficial for the diagnosis of mycobacterial infections. The turnaround time for test results is much more rapid than for specialist mycobacterial culture, which can take 6–8 weeks for an *M. bovis* diagnosis and three months or longer to get a positive result for *M. microti* (O'Halloran and Gunn-Moore, 2017), which is the most commonly cultured *Mycobacterium* species from cats in Great Britain (Gunn-Moore et al., 2011). As the IGRA only requires 2 mL of heparinised blood it is less invasive than sampling some lesions (O'Halloran et al., 2019; Stavinohova et al., 2019) and it does not require the use of general anaesthesia. Additionally, it is the only test available for use in cats that have no discernible lesions to sample, and it has been used as a screening tool (O'Halloran et al., 2019). It is, however, dependent on the viability of isolated PBMCs; proper handling and storage of samples prior to testing is essential to minimise the risk of false-negative results.

Wide-spread use of the IGRA for diagnosing TB in cattle has allowed for studies to determine the diagnostic accuracy of this assay in populations where the prevalence of disease varies (Aagaard et al., 2006; Antognoli et al., 2011; Faye et al., 2011), the impact of prior tuberculin skin testing (Thom et al., 2004, 2006) and the role of prior exposure or infection with non-tuberculous mycobacteria (NTM) (Hope et al., 2005; Jenkins et al., 2018). This has helped to establish decisional cut-off values to maximise the sensitivity and specificity of the bovine IGRA, depending on the question being asked of the test (de La Rua-Domenech et al., 2006). Current thresholds for antigen positivity and tuberculin-bias in cats are in-line with those used for cattle (Vordermeier et al., 2001; de La Rua-Domenech et al., 2006; Schiller et al., 2009); however, the validity of these thresholds in cats has never been statistically tested due to the lack of sufficient defined sample cohorts. Previous studies in cats have focused on assessing individual-animal responses rather than using pre-determined decisional cut-off values (Rhodes et al., 2008a, 2011).

The aims of this study were to statistically determine the diagnostic accuracy of the feline IGRA under current interpretive guidelines, explore and establish new thresholds for antigen positivity and tuberculin-bias to improve test sensitivity and/or specificity, and determine the degree of agreement between IGRA results and those obtained from culture and/or polymerase chain reaction (PCR) tests. A secondary aim was to explore test quality control (QC) failures to identify factors that could be addressed to reduce the test failure rate.

## 2. Materials and methods

### 2.1. Ethical statement

Ethical approval for this study was granted by the institutional Veterinary Ethical Review Committee (approval no. 79 14).

### 2.2. Study populations

A retrospective analysis of IGRA results was undertaken, using data from cats tested for mycobacterial disease by Biobest Laboratories, Edinburgh between May 2013 and October 2019. Each assay had been performed as previously described (Rhodes et al., 2008a, 2011). All samples were transported to the testing lab at ambient temperature. Once the sample had arrived, PBMCs were isolated from the 2 mL heparinised blood samples following a 1:1 dilution with Dulbecco's Phosphate-Buffered Saline (DPBS, Sigma, UK) and layering over Histopaque 1077 (Sigma, UK), followed by centrifugation at 800 x *g* for 40 min at room temperature. PBMCs were removed and washed by re-suspending in DPBS and centrifuging at 999 x *g* for 10 min at room temperature, counted with Trypan Blue dye exclusion to assess cell integrity and therefore viability, and re-suspended in complete culture media (RPMI 1640 with 100 μg/mL L-glutamine, 10 % foetal bovine serum, 100 μg/mL penicillin, 100 U/mL streptomycin, $5 \times 10^{-5}$ mol/L 2-mercaptoethanol and non-essential amino acids) to a concentration of $2 \times 10^{6}$ cells/mL. For each test condition, 100 μL of PBMC suspension was stimulated in duplicate with an equal volume of PPDA or PPDB (Lelystad, Prionics, Netherlands) at a final concentration of 10 μg/mL, ESAT-6/CFP-10 (Lionex, Germany) at a final concentration of 5 μg/mL, and the phorbol myristate acetate-calcium ionophore (PMA-Ca, Sigma, UK) positive control at a final concentration of 50 ng/mL and 1 μg/mL, respectively. The negative control consisted of unstimulated PBMCs in complete culture media. Following incubation at 37 °C in 5% $CO_2$ for four days, the duplicate supernatants were removed and pooled for IFNγ quantification by ELISA. Cell culture supernatants were removed and either tested immediately or frozen at −20 °C for future testing if required. A commercial ELISA kit for the quantitative determination of feline IFNγ in cell culture supernatants was performed following the manufacturer's instructions (GenWay Biotech, USA). Test submission data and results were cross-referenced with clinical data supplied by the referring veterinary surgeon (RVS) to the authors for case advice and management. This was done in line with owner consent, and the RVS was contacted for further clinical information, where necessary.

From the 594 individual cats that had undergone IGRA testing, records were consulted to identify those that had a culture- or PCR-confirmed diagnosis of mycobacterial disease, undertaken at private commercial testing centres or as part of state-funded investigations. These animals were used as the positive control cohort to estimate test sensitivity for diagnosing all mycobacterial infections, as well as test sensitivity for individual pathogens or complexes (*M. bovis*, *M. microti*, MTBC and NTM). The negative control cohort consisted of cats that were IGRA tested either on suspicion of, or to rule out mycobacterial disease, but ultimately had a final diagnosis of something other than mycobacteriosis. Further diagnostics appropriate for each case, including mycobacterial culture or PCR, were performed at the discretion of the RVS. These animals were used to estimate test specificity.

### 2.3. Current test interpretation and analysis

Results of IGRA testing were classified according to current recommendations (see below). For a test to pass, the coefficient of variation (CV) for replicated control and test wells had to be less than 30 %. The positive control (PC) $OD_{PC}$ value had to be $\geq$ 0.40 and exceed the negative control (NC) $OD_{NC}$ value by 0.10. The $OD_{NC}$ value had to be $\leq$ 0.30. To determine positivity to mycobacterial antigens the OD value for each antigen (PPDA, PPDB and ESAT-6/CFP-10) had to exceed the $OD_{NC}$ by 0.10. Tuberculin-bias was interpreted as the OD value for one PPD exceeding the other PPD OD value by 0.10 (Schiller et al., 2009).

Tests that passed were interpreted in accordance with previously established criteria for patterns of antigen positivity and tuberculin-bias *i.e.* MTBC = PPDB > PPDA, NTM = PPDA > PPDB. MTBC results were then categorised based on the ESAT-6/CFP-10 antigen response *i.e. M. bovis* = PPDB > PPDA, ESAT-6/CFP-10-positive; *M. microti* = PPDB > PPDA, ESAT-6/CFP-10-negative (Rhodes et al., 2011). Where there was a positive result to both PPD antigens, but the difference between two OD PPD values was less than 0.10 *i.e.* PPDA = PPDB, the result was recorded as equivocal *e.g.* an OD value of 0.30 for PPDA (positive) and 0.33 for PPDB (positive) with no PPD-bias (0.03). Results with positivity to one PPD antigen, but no tuberculin-bias *i.e.* the OD value for one, but not both PPD antigens exceeded the $OD_{NC}$ by $\geq$ 0.10, and the difference between the two OD PPD values was less than 0.10, were ascribed as borderline *e.g.* an OD value of 0.12 for PPDA (positive) and 0.04 for PPDB (negative) has no clear PPD-bias (0.08). Test sensitivity for each group (MTBC or NTM) or pathogen (*M. bovis* or *M. microti*), according to the above interpretations, were calculated for cats with culture- or PCR-diagnosed mycobacterial disease *i.e.* for cats with *M. bovis* infection, PPDB > PPDA, ESAT-6/CFP-10 positive results were compared against all other interpretations to determine sensitivity. The overall sensitivity of the IGRA to give any positive response in cases of mycobacterial infection was also calculated. Test specificity was determined by applying these test result interpretations to the negative control cohort of cats. Positive and negative likelihood ratios (LR + and LR-) were calculated for the ability of the IGRA to give a positive result in a cat with mycobacterial infection, and to give a negative result in cases of non-mycobacterial disease. Test sensitivity, specificity and LRs were calculated using GraphPad Prism version 9.0.0 for Windows, GraphPad Software, San Diego, California, USA.

### 2.4. Positive and negative control threshold assessment

An expanded dataset of 741 IGRA tests, totalling all tests performed on the 594 individual cats between May 2013 and October 2019, was used to establish PC and NC thresholds; 107 cats underwent repeat IGRA testing, either due to test failures or serial monitoring during- and post-treatment. Test results where the CV exceeded 30 % were excluded, as were those where the duration between obtaining the sample and processing this for IGRA exceeded 14 days. The $OD_{NC}$ cut-off value was established as the mean $OD_{NC}$ plus two standard deviations (SD); all sample NCs should be below this value to pass test QC. The $OD_{PC}$ value was calculated as the mean $OD_{NC}$ plus three standard deviations; all sample PCs should be above this value to pass test QC. Comparison of $OD_{PC}$-$OD_{NC}$ values between cats where the $OD_{PC}$ passed and where the $OD_{PC}$ failed was undertaken to identify any overlap between tests where the $OD_{PC}$ failed yet was substantially greater than the $OD_{NC}$.

Possible factors, namely the signalment (age, gender and breed of the cat), season and sample processing time, to explain failing $OD_{PC}$ values were explored with univariate binomial logistic regression. Any factors approaching statistical significance ($p < 0.20$) were included in a multivariate model along with potential confounders followed by multidirectional stepwise selection to reduce the model to its simplest components without compromising model performance. Model goodness-of-fit was determined using the Hosmer-Lemeshow test. Statistical significance in the multivariate model was set at $p < 0.05$. Logistic regression was performed using RStudio (RStudio Team, 2018).

### 2.5. Antigen threshold exploration

Newly established PC and NC thresholds were applied to the positive and negative cohorts, and test results identified. Receiver operator characteristic (ROC) curves were constructed for each antigen (PPDB, PPDA and ESAT-6/CFP-10) and for PPD-bias (both PPDA-PPDB and PPDB-PPDA) to determine the area under the curve (AUC), and explore different cut-off values to improve the sensitivity and specificity for each scenario (Swets, 1988). For all ROC curves the non-mycobacterial disease cases were used as the negative group. All cases of mycobacterial disease were used for constructing the PPDA ROC curve. The PPDA-bias curve was constructed using cases diagnosed with NTM infections. For the PPDB and PPDB-bias curves, only cases diagnosed as *M. bovis*, *M. microti* or unclassified MTBC were used. Finally, ESAT-6/CFP-10 ROC curves were constructed using cases of *M. bovis* infection, and cases of infection with any RD-1-positive *Mycobacterium* species. Proposed new cut-offs were identified where appropriate and applied to the mycobacterial disease and negative control groups to show within-group sensitivity, specificity and LR values. Construction and analysis of ROC curves, and calculation of test sensitivity, specificity and likelihood ratios were performed using GraphPad Prism 9.0.0.

### 2.6. Test agreement analysis

The agreement between IGRA results using current and proposed new thresholds against culture/PCR tests was calculated for cats diagnosed with *M. bovis*, *M. microti* or an NTM infection as well as non-mycobacterial disease *i.e.* negative control cats; cats with unclassified MTBC infections were excluded from the analysis due to the overlap with *M. bovis* and *M. microti*. The kappa coefficients for each group and overall percentage agreement were determined and compared for the two interpretations (McHugh, 2012). Cohen's kappa was calculated using Minitab 17, State College, Pennsylvania, USA.

## 3. Results

### 3.1. Study population

A total of 117 defined individuals were identified for further analysis: 80 cats had a culture or PCR-confirmed diagnosis of mycobacterial disease (Supplemental Table 1). An MTBC infection was identified in 70 cats and compared to 10 with NTM. Of the MTBC infections, 36 cats were diagnosed with *M. bovis* and 12 diagnosed with *M. microti*; the remaining 22 cats had an MTBC infection which could not be further classified (either due to insufficient DNA for MTBC PCR speciation, lack of test availability to differentiate members of the MTBC, or financial restrictions). Among the NTM, four cats were diagnosed with *M. avium*-complex, two with *M. kansasii*, two with *M. malmoense*, one with *M. abscesuss-chelonae*-complex and one with *M. chelonae*. Retroviral testing *i.e.* feline leukaemia virus (FeLV) antigen and feline immunodeficiency virus (FIV) antibody was performed on 33 cats, one of which was positive for FIV. The negative control cohort comprised 37 cats (Table 1). Three of these cats were tested by PCR for *Mycobacterium* species and were negative (two cats had eosinophilic/neutrophilic/mixed airway disease and one cat had *Actinomyces* species infection). Ziehl-Neelsen staining on 11 of the 17 cats with non-mycobacterial respiratory disease was negative (ZN staining was not performed for the remaining six cats). Five of the 11 cats with neoplastic disease also underwent ZN staining; these were all negative. Fourteen cats were tested for retroviruses and all were negative.

### 3.2. Current test interpretation and analysis

Of the 117 individuals identified for analysis above, the CV exceeded

**Table 1**
Diagnosis of cats that underwent interferon-gamma (IFNγ) release assay (IGRA) testing that were ultimately diagnosed with a non-mycobacterial disease. Mycobacterial PCR was undertaken on two cats with eosinophilic/neutrophilic/mixed airway disease, and one cat that was PCR-positive for *Actinomyces* infection; all three were negative. Mycobacterial culture was not performed for any cat. Additional diagnostics were performed as deemed clinically appropriate by the referring veterinary surgeon.

| Diagnosis | No. of cats |
|---|---|
| *Respiratory Disease (excluding neoplasia)* | *17* |
| - Eosinophilic/neutrophilic/mixed airway disease | - 8 |
| - Bacterial pneumonia | - 3 |
| - Idiopathic pulmonary fibrosis | - 3 |
| - Pulmonary abscess | - 2 |
| - Foreign body-associated pyogranulomatous inflammation | - 1 |
| *Neoplasia* | *11* |
| - Intestinal lymphoma | - 2 |
| - Bile duct carcinoma | - 1 |
| - Pulmonary adenocarcinoma | - 1 |
| - Soft tissue sarcoma | - 1 |
| - Thymoma | - 1 |
| - Unclassified pulmonary neoplasia | - 3 |
| - Unclassified mediastinal epithelial neoplasia | - 2 |
| *Infectious Disease* | *5* |
| - *Actinomyces* spp./*Nocardia* spp. | - 3 |
| - Feline Infectious Peritonitis | - 1 |
| - Toxoplasmosis (Central Nervous System) | - 1 |
| *Other* | *4* |
| - Acne dermatitis | - 1 |
| - Idiopathic anterior uveitis | - 1 |
| - Peritoneal-pericardial diaphragmatic hernia | - 1 |
| - Sterile peritonitis | - 1 |
| **Total** | **37** |

30 % in 8 of the 585 test conditions (PC, NC, PPDA, PPDB and ESAT-6/CFP-10 for each cat). This equated to five cats *i.e.* two cats had multiple test conditions that failed. In addition, the $OD_{PC}$ value was less than 0.40 for 20 cats (this includes two cats that also had CV failures), whereas the $OD_{NC}$ value exceeded 0.30 for two cats. A total of 25 cats (21.4 %) failed the $OD_{PC}$, $OD_{NC}$ and/or CV thresholds. The failed tests came from 15 cats in the mycobacteria-positive group and 10 cats in the negative control group; these were all removed from subsequent analysis.

The IGRA result from each cat that passed the PC, NC and CV thresholds (n = 92) was interpreted in accordance with previously established guidelines (Rhodes et al., 2011) (Table 2). A PPDB-biased response was observed in 84.4 %, 90.0 % and 76.5 % of *M. bovis*, *M. microti* and unclassified MTBC infections respectively; a PPDA-biased response was identified in 10.0 % of cats with *M. microti* (n = 1), while an equivalent PPD-response was recorded in 6.3 % of *M. bovis*-positive cats (n = 2). Only 43.8 % of cats with *M. bovis* (n = 14) gave a positive result for ESAT-6/CFP-10, including five of nine cats fed the commercial raw food diet associated with an outbreak of *M. bovis* TB (O'Halloran et al., 2019, 2020). A negative IGRA result was recorded in 3.1 % (n = 1) and 17.6 % (n = 3) of cats with *M. bovis* and unclassified MTBC infections, respectively, giving a total of 6.8 % negative (n = 4) for all MTBC infections. One IGRA-negative cat had only multiple cutaneous lesions, whereas the three remaining cats had evidence of systemic disease, including two cats implicated in the raw food TB outbreak; all three having pulmonic lesions on thoracic imaging, with one also having an abdominal mass and tracheobronchial lymphadenopathy, while the other two had cutaneous lesions with local lymphadenopathy.

Among the NTM, a PPDA-biased response was identified in both cats infected with *M. avium*, the sole cat with *M. malmoense* infection and one cat diagnosed with *M. kansasii*; the other *M. kansasii*-positive cat had an equivalent PPD-response. These five individuals did not give a positive result to ESAT-6/CFP-10. The cat diagnosed with *M. chelonae* gave an IGRA result strongly suggestive of *M. bovis* infection *i.e.* PPDB-biased and ESAT-6/CFP-10 positive, either indicating a true test false-positive or a potential undisclosed *M. bovis* co-infection.

Two cats in the negative control group gave a PPDA-biased response; these cats were diagnosed with actinomycosis, and toxoplasmosis along with exocrine pancreatic insufficiency, respectively. A third cat gave a positive response to ESAT-6/CFP-10 but was negative for both PPD antigens and was diagnosed at *post-mortem* examination with primary intestinal lymphoma. The remaining 88.9 % of 'negative' cats (n = 24) were IGRA negative.

Test positivity using the current test cut-offs and interpretation is shown for each infected cohort in Table 3. Overall sensitivity of the IGRA for any mycobacterial infection was 93.9 %, and the specificity was 88.9 %. For all MTBC infections the sensitivity was 83.1 % and specificity was 100 %, whereas for the NTM the sensitivity was 66.7 % and specificity was 92.6 %. Among the MTBC, test sensitivity for *M. bovis* was 43.8 %, compared to 90.0 % for *M. microti.* The LR + for a positive IGRA result in confirmed-cases of mycobacterial infection was calculated as 8.45, and the LR- as 0.07 *i.e.* the post-test odds of disease are approximately 8.5 times the pre-test odds of disease if the IGRA is positive, whereas the post-test odds of disease are one-fourteenth the pre-test odds given a negative IGRA result, respectively.

**Table 3**
Sensitivity and specificity values, with 95 % confidence intervals for cats with different mycobacterial infections against 'negative' cats.

| | Sensitivity | 95 % CI | Specificity | 95 % CI |
|---|---|---|---|---|
| **MTBC and NTM (n = 65)** | 93.9 | 85.2–97.6 | 88.9 | 71.9–96.1 |
| **All MTBC (n = 59)** | 83.1 | 71.5-90.5 | 100.0 | 87.5-100.0 |
| - ***M. bovis*(n = 32)** | - 43.8<br>- 90.0 | - 28.2–60.7<br>- 59.6-99.5 | - 100.0<br>- 100.0 | - 87.5-100.0 |
| - ***M. microti*(n = 10)** | | | | - 87.5-100.0 |
| **NTM (n = 6)** | 66.7 | 30.0–94.1 | 92.6 | 76.6–98.7 |

MTBC = *Mycobacterium tuberculosis*-complex. NTM = non-tuberculous mycobacteria. CI = confidence interval.

**Table 2**
Interferon-gamma positive response rates (%) in cats according to different test criteria. An equivocal result was recorded in cases where there was positivity to both PPDA and PPDB antigens, but there was no tuberculin bias *i.e.* the difference in the optical density values was less than 0.10. Borderline results were recorded in cases of positivity to one PPD antigen, but not both, and there was no tuberculin bias.

| | B > A | E + | B > A and E + | A > B | Equivocal (A = B) | Negative | Borderline |
|---|---|---|---|---|---|---|---|
| ***M. bovis* (n = 32)** | 84.4 | 43.8 | 43.8 | 0.0 | 6.3 | 3.1 | 6.3 |
| ***M. microti* (n = 10)** | 90.0 | 0.0 | 0.0 | 10.0 | 0.0 | 0.0 | 0.0 |
| **Unclassified MTBC (n = 17)** | 76.5 | 17.6 | 17.6 | 0.0 | 0.0 | 17.6 | 5.9 |
| ***M. avium* (n = 2)** | 0.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 | 0.0 |
| ***M. kansasii* (n = 2)** | 0.0 | 0.0 | 0.0 | 50.0 | 50.0 | 0.0 | 0.0 |
| ***M. chelonae* (n = 1)** | 100.0 | 100.0 | 100.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| ***M. malmoense* (n = 1)** | 0.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 | 0.0 |
| **'Negative' (n = 27)** | 0.0 | 3.7 | 0.0 | 7.4 | 0.0 | 88.9 | 0.0 |

MTBC = *Mycobacterium tuberculosis*-complex. A = PPDA. B = PPDB. E = ESAT-6/CFP-10.

### 3.3. Re-assessment of positive and negative quality control thresholds

From the set of 741 IGRA tests, 737 were processed within 14 days of the sample being taken. Of these, 22.8 % of tests (n = 168) failed the $OD_{PC}$ threshold compared to 2.6 % (n = 19) exceeding the $OD_{NC}$ threshold. The CV exceeded 30 % in 1.2 % of positive or control wells (n = 17). In total, 26.7 % of tests (n = 197) failed $OD_{PC}$, $OD_{NC}$ and/or CV thresholds, the largest group being $OD_{PC}$-only failures (22.1 %, n = 163).

The mean $OD_{NC}$ value was calculated from the tests where the CV did not exceed 30 % (n = 730). The mean $OD_{NC}$ was 0.09 and the negative control threshold was established as 0.28 (mean $OD_{NC}$ plus 2SD); this was rounded to 0.30 for ease of reporting. The positive control was established as 0.38 (mean $OD_{NC}$ plus 3SD), which was also rounded to 0.40 for reporting ease. These values were consistent with currently established thresholds.

For all tests with a passing $OD_{PC}$ value *i.e.* $OD_{PC} \geq 0.40$, and a CV $\leq$ 30 % for both PC and NC (n = 558), the average difference between the $OD_{PC}$ and the $OD_{NC}$ was 2.01 (range 0.32–5.01); this value ranged from -0.12 to 0.36 for those with a failing $OD_{PC}$ *i.e.* $OD_{PC} < 0.40$ (but PC and NC CV $\leq$ 30 %) (n = 163), indicating some overlap between the $OD_{PC}$-$OD_{NC}$ values for tests where the $OD_{PC}$ passes or fails. Data were explored for cats where $OD_{PC} < 0.40$ and there appeared to be reasonable distinction between $OD_{PC}$ and $OD_{NC}$ values to make meaningful interpretation of results when the difference was $\geq$ 0.20 *i.e.* at least two standard deviations above the $OD_{NC}$ value calculated from the larger subset of data. Therefore, the proposed new PC and NC thresholds were decided as; $OD_{PC} \geq 0.40$; $OD_{NC} \leq 0.30$ and $OD_{PC}$-$OD_{NC} \geq 0.10$; if the $OD_{PC} < 0.40$, the test can be considered 'acceptable' provided the $OD_{PC}$-$OD_{NC} \geq 0.20$.

Factors to explain PC failure (where $OD_{PC} < 0.40$ but CV $\leq$ 30 %) in a univariate model were time from sampling to the IGRA test being performed, the season when the cat was sampled as a potential indicator of sample transit temperature (December to February = Winter, March to May = Spring, June to August = Summer, September to November = Autumn), and the age, gender (male *vs* female) and breed (domestic short, medium or longhair [DxH] *vs* non-DxH) of the cat. Only tests with complete data were included (n = 608), of which 462 tests passed and 146 tests failed. Over 93 % of tests were performed on samples taken within the preceding 48 h, in accordance with current recommendations. Test PC fails during spring, summer, autumn and winter sample collections were 28.7 %, 26.1 %, 16.4 % and 23.1 % respectively (Table 4). Univariate analysis identified statistically significant differences between the number of tests passing PC thresholds in spring and summer compared to autumn, with fewer tests passing in spring (p = 0.014, odds ratio [OR] 0.49 [0.28−0.86]) and summer (p = 0.042, OR 0.56 [0.31−0.98]). The season of sampling, cat breed and duration from sampling to testing were then included in a multivariate model, with age and gender added as potential confounders. Multidirectional stepwise selection removed age, gender and duration from the multivariate model, which supported the previous findings of an effect of season on PC performance, with PC failures more likely to occur if the cat was sampled in spring (p = 0.010, OR 0.47 [0.27−0.83]) or summer (p = 0.028, OR 0.53 [0.30−0.93]) compared to autumn, but also found that non-DxH cats were less likely to have PC failures (p = 0.041, OR 1.55 [1.02−2.36]).

### 3.4. Reassessment of antigen cut-off values

The proposed new PC and NC thresholds were applied to the subset of 117 cats with either a confirmed mycobacterial or non-mycobacterial disease; the total number of QC fails dropped to 17.1 % (n = 20). From the original data exploration, two additional cats with unclassified MTBC infections were included for subsequent analysis, along with three negative control cats.

Construction of ROC curves for these expanded groups showed outstanding performance *i.e.* AUC > 0.900 for NC-corrected PPDA and PPDB, as well as for determining PPDB-bias. The AUC for ESAT-6/CFP-10 (0.896) was interpreted as excellent, as was determining PPDA-bias in cats with mycobacterial disease (0.800), although the 95 % CI for this was very wide. These ROC curves provided cut-off values for each antigen and for comparative PPD-bias (Table 5). The sensitivity of all antigens was enhanced by small reductions in test cut-off values, whilst maintaining high specificity: PPDA sensitivity across all mycobacterial infections was improved from 74.6%–83.6% by reducing the cut-off value from 0.10 to 0.07, (specificity maintained at 93.3 %). PPDB sensitivity in cases of MTBC infection marginally increased from 90.2%–91.8% by reducing the cut-off value from 0.1 to 0.07 (specificity maintained at 100 %). ESAT-6/CFP-10 sensitivity was increased from 43.8%–68.8% (in cases of *M. bovis* infection) and 41.2%–67.7% (in cases of infection with RD-1 positive mycobacteria [in this study, *M. bovis* and *M. kansasii*]) by reducing the cut-off value from 0.10 to 0.05 (both with specificity maintained at 96.7 %).

Similarly, PPDB-bias sensitivity (MTBC infections) was improved from 82.0%–93.4% by reducing the cut-off from 0.10 to 0.05 (specificity maintained at 100 %); and dropping the PPDA-bias cut-off (NTM infections) from 0.10 to 0.05 improved sensitivity from 66.7%–83.3% (specificity maintained at 93.3 %).

Applying these new cut-offs to the test interpretations of the 67 mycobacterial-infected and 30 negative control cats allowed for classification of test results according to the pattern of results (Table 6) and calculation of new sensitivity and specificity parameters within each infection group (Table 7, compare with Table 3). Overall sensitivity for MTBC infections increased from 83.1%–90.2%, while specificity was maintained at 100 %. Within the MTBC, the sensitivity for *M. bovis* infections increased from 43.8%–68.8%. The LR + marginally increased from 8.45 to 9.40 *i.e.* the post-test odds of disease are almost 9.5-times the pre-test odds given a positive IGRA result, whereas the LR- remained at 0.07.

**Table 4**
Univariate and reduced multivariate binomial logistic regression analysis for positive control failures. Goodness-of-fit analysis showed insufficient evidence to conclude the models did not fit the data (not shown).

| | | Univariate | | | Multivariate | | |
|---|---|---|---|---|---|---|---|
| Variable | Summary Statistics | Odds Ratio | 95 % CI | p-value | Odds Ratio | 95 % CI | p-value |
| Season | Autumn: 134 (Pass: 112, Fail: 22) | *Reference* | – | – | *Reference* | – | – |
| | Spring: 164 (Pass: 117, Fail: 47) | 0.49 | 0.28−0.86 | 0.014 | 0.47 | 0.27−0.83 | 0.010 |
| | Summer: 176 (Pass: 130, Fail: 46) | 0.56 | 0.31−0.98 | 0.042 | 0.53 | 0.30−0.93 | 0.028 |
| | Winter: 134 (Pass: 103, Fail: 31) | 0.65 | 0.36−1.20 | 0.169 | 0.64 | 0.35−1.18 | 0.156 |
| Breed | DxH: 412 (Pass: 304, Fail: 108) | *Reference* | – | – | *Reference* | – | – |
| | Non-DxH: 196 (Pass: 158, Fail: 38) | 1.48 | 0.97−2.24 | 0.067 | 1.55 | 1.02−2.36 | 0.041 |
| Duration | 2 days (0–13 days) | 0.92 | 0.82−1.03 | 0.148 | – | – | – |
| Gender | Female: 254 (Pass: 189, Fail: 65) | *Reference* | – | – | – | – | – |
| | Male: 354 (Pass: 273, Fail: 81) | 1.16 | 0.80−1.69 | 0.441 | – | – | – |
| Age | 6 years (2 months – 17.5 years) | 0.99 | 0.94−1.04 | 0.710 | – | – | – |

CI = confidence interval. DxH = domestic short, medium or longhair.

**Table 5**
Results of receiver operator characteristic (ROC) curve analysis using current thresholds and the corresponding sensitivity and specificity values. New cut-off values for certain criteria are proposed, with new sensitivity and specificity values quoted.

| | Cut-off | AUC | 95 % CI | Se | 95 % CI | Sp | 95 % CI | New cut-off | Se | 95 % CI | Sp | 95 % CI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **PPDA - NC** | 0.10 | 0.913 | 0.854–0.972 | 74.6 | 63.1–83.5 | 93.3 | 78.7–98.8 | 0.07 | 83.6 | 72.9–90.6 | 93.3 | 78.7–98.8 |
| **PPDB - NC** | 0.10 | 0.991 | 0.976–1.000 | 90.2 | 80.2–95.4 | 100.0 | 88.7–100.0 | 0.07 | 91.8 | 82.2–96.5 | 100.0 | 88.7–100.0 |
| **E6C10*M.bovis* - NC** | 0.10 | 0.896 | 0.819–0.973 | 43.8 | 28.2–60.7 | 96.7 | 83.3–99.8 | 0.05 | 68.8 | 51.4–82.1 | 96.7 | 83.3–99.8 |
| **E6C10$^{RD-1}$ - NC** | 0.10 | 0.899 | 0.823–0.974 | 41.2 | 26.4–57.8 | 96.7 | 83.3–99.8 | 0.05 | 67.7 | 50.8–80.9 | 96.7 | 83.3–99.8 |
| **PPDB - PPDA** | 0.10 | 0.965 | 0.921–1.000 | 82.0 | 70.5–89.6 | 100.0 | 88.7–100.0 | 0.05 | 93.4 | 84.3–97.4 | 100.0 | 88.7–100.0 |
| **PPDA - PPDB** | 0.10 | 0.800 | 0.509–1.000 | 66.7 | 30.0–94.1 | 93.3 | 78.7–98.8 | 0.05 | 83.3 | 43.7–99.2 | 93.3 | 78.7–98.8 |

PPDA = purified protein derivative from *M. avium*; PPDB = purified protein derivative from *M. bovis*; E6C10$^{M.bovis}$ = early secreted antigenic target 6 kDa-culture filtrate protein 10 kDa for cases of *M. bovis*; E6C10$^{RD-1}$ = early secreted antigenic target 6 kDa-culture filtrate protein 10 kDa for cases of infection with region of difference-1 positive *Mycobacteria* (*M. bovis* and *M. kansasii*); NC = negative control. AUC = area under the curve. CI = confidence interval. Se = sensitivity. Sp = specificity.

**Table 6**
Interferon-gamma positive response rates (%) in cats according to different test criteria. Compared to Table 2, no results were interpreted as equivocal (positivity to both PPD antigens, but with a lack of PPD bias) or borderline (positivity to one, but not both PPD antigens, and with no PPD bias).

| | B > A | E + | B > A and E + | A > B | A > B and E + | Negative |
|---|---|---|---|---|---|---|
| ***M. bovis* (n = 32)** | 93.8 | 68.8 | 68.8 | 3.1 | 0.0 | 3.1 |
| ***M. microti* (n = 10)** | 90.0 | 0.0 | 0.0 | 10.0 | 0.0 | 0.0 |
| **Unclassified MTBC (n = 19)** | 84.2 | 21.1 | 21.1 | 0.0 | 0.0 | 15.8 |
| ***M. avium* (n = 2)** | 0.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 |
| ***M. kansasii* (n = 2)** | 0.0 | 50.0 | 0.0 | 100.0 | 50.0 | 0.0 |
| ***M. chelonae* (n = 1)** | 100.0 | 100.0 | 100.0 | 0.0 | 0.0 | 0.0 |
| ***M. malmoense* (n = 1)** | 0.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 |
| **'Negative' (n = 30)** | 0.0 | 3.3 | 0.0 | 6.7 | 0.0 | 90.0 |

MTBC = *Mycobacterium tuberculosis*-complex. A = PPDA. B = PPDB. E = ESAT-6/CFP-10.

**Table 7**
Updated sensitivity and specificity values with 95 % confidence intervals using new cut-off values for interferon-gamma release assay result interpretation.

| | Sensitivity | 95 % CI | Specificity | 95 % CI |
|---|---|---|---|---|
| **MTBC and NTM (n = 67)** | 94.0 | 85.7–97.7 | 90.0 | 74.4–96.5 |
| **All MTBC (n = 61)** | 90.2 - 68.8 | 80.2–95.4 - 51.4–82.1 | 100.0 - 100.0 | 88.7–100.0 - 88.7–100.0 |
| - ***M. bovis*(n = 32)** | - 90.0 | - 59.6-99.5 | - 100.0 | - 88.7–100.0 |
| - ***M. microti*(n = 10)** | | | | |
| **NTM (n = 6)** | 66.7 | 30.0–94.1 | 93.3 | 78.7–98.8 |

MTBC = *Mycobacterium tuberculosis*-complex. NTM = non-tuberculous mycobacteria. CI = confidence interval.

One cat with *M. bovis* infection remained IGRA negative following test reassessment, as did two cats with unclassified MTBC infection. A third cat with unclassified MTBC infection initially failed the IGRA ($OD_{PC} = 0.30$, $OD_{NC} = 0.05$) but now tested IGRA negative; this cat had systemic mycobacterial disease. However, one previous MTBC-infected cat that had tested IGRA negative now gave a PPDB-biased response ($OD_{NC}$ corrected values, $OD_{PPDA} = 0.02$, $OD_{PPDB} = 0.08$, $OD_{E6C10} = 0.00$). Two *M. bovis*-positive cats that initially had equivalent PPD-responses showed PPDB-bias after applying the new thresholds, as did one cat that previously gave a borderline response (PPDB-positive, but no PPD-bias). The second borderline cat (previously PPDA-positive without PPD-bias) now gave a PPDA-biased result without positivity to ESAT-6/CFP-10. Among the NTM, one *M. kansasii*-positive cat now showed a PPDA-biased response in addition to positivity to the RD-1 proteins ESAT-6/CFP-10, having initially been PPD-equivalent and ESAT-6/CFP-10 negative. The results in the negative control group did not change with the new thresholds applied; the additional three cats included after changing the test pass criteria all tested negative.

### 3.5. Test agreement analysis

Overall agreement between IGRA and culture/PCR, as well as kappa coefficient values, were calculated for the current and proposed new IGRA thresholds. Using current cut-off values there was 68.0 % agreement (95 % CI: 56.2–78.3 %; 51/75) between IGRA and culture/PCR; this increased to 79.5 % (95 % CI: 68.8–87.8 %; 62/78) under proposed new thresholds. The kappa coefficient was considered weak for *M. bovis* ($\kappa = 0.44$), *M. microti* ($\kappa = 0.46$) and NTM ($\kappa = 0.58$) infections under current thresholds; the value of kappa increased for *M. bovis* ($\kappa = 0.70$) and *M. microti* ($\kappa = 0.60$) infections using new cut-offs to give moderate agreement, but for NTM infections the value of kappa decreased slightly ($\kappa = 0.53$). For the negative control cats there was a marginal increase in the value of kappa between the current ($\kappa = 0.88$) and new ($\kappa = 0.89$) IGRA thresholds, with agreement remaining strong. Overall, the kappa agreement increased from weak ($\kappa = 0.57$) to moderate ($\kappa = 0.71$).

## 4. Discussion

The aim of this study was to statistically evaluate the performance of the feline IGRA to accurately diagnose mycobacterial infections in domestic cats within the clinical setting, and in doing so to modify the decisional cut-off values to improve test performance. This study has entailed the investigation of individual cat test data accumulated over a period of six years, only now providing sufficient numbers for meaningful analysis. Our data show that small adjustments (reductions) of the cut-off values for PPDA, PPDB and ESAT-6/CFP-10 antigen and PPD-bias measurements improved the overall test sensitivity without compromising specificity, and with a notable improvement in the sensitivity for diagnosing infection with *M. bovis*. Altering these thresholds also improved test agreement between IGRA and culture/PCR.

Tuberculosis is increasingly recognised in cats, with strong regionalisation of *M. bovis*- and *M. microti*-positive culture submissions from high- and low-risk areas of the UK respectively (Gunn-Moore et al., 2011; More et al., 2018). This study showed that current thresholds used for interpreting the IGRA risks underdiagnosing cases of *M. bovis* in cats (false-negatives) and/or that they may be misinterpreted as infection with *M. microti*. Infection with members of the MTBC should induce a PPDB-biased response in cats (Rhodes et al., 2008a, 2011), and inclusion of the antigenic cocktail ESAT-6/CFP-10 is intended to differentiate between infection with *M. bovis* and *M. microti* (Rhodes et al., 2008b), as RD-1 is deleted in *M. microti* (Pym et al., 2002; Frota et al., 2004). This is important because there is greater zoonotic risk from feline *M. bovis* infections than from infections with *M. microti* (Human Animal

Infections and Risk Surveillance (HAIRS) group, 2014; O'Connor et al., 2019) and these may also play a role in the greater ecological epidemiology of bovine TB (Monies et al., 2000; Gunn-Moore et al., 2011). Based on studies of cattle, approximately 82 % of animals infected with *M. bovis* will generate a positive IGRA response to ESAT-6/CFP-10 (Buddle et al., 2001; Vordermeier et al., 2001, 2016; Nuñez-Garcia et al., 2018); this study showed positivity in cats to this antigenic cocktail was only 43.8 %. In other species it has been shown that IFNγ secretion by T-lymphocytes in response to ESAT-6/CFP-10 stimulation slowly peaks and then declines, but it can fluctuate (Parsons et al., 2017). This dynamic pattern of IFNγ secretion may explain why some cats infected with *M. bovis* were negative to this antigen; if mycobacterial disease was not considered early on in the diagnostic investigation this would result in delays to performing an IGRA, by which point it may be too late to detect a substantial IFNγ response to ESAT-6/CFP-10. This decline in T-lymphocyte response to ESAT-6/CFP-10 stimulation, in addition to the hypothesised timeline of infection several months prior to testing, may partially explain why some of the cats that had been fed the commercial raw food diet associated with an outbreak of *M. bovis* TB were ESAT-6/CFP-10 negative despite a culture or PCR-confirmed diagnosis (O'Halloran et al., 2019, 2020). The incorporation of other test-antigens specific to *M. bovis* could be considered in future feline IGRA testing to help improve the performance of the test. For example, studies in cattle have shown immune responses to antigen cocktails that include peptides from Rv3019c (ESAT-6 like protein 9) and Rv0288 (CFP-7), may enhance sensitivity of earlier detection, allowing more rapid identification of infected animals (Cockle et al., 2006). Although this study suggests reducing the threshold for ESAT-6/CFP-10 positivity in cats to improve sensitivity for *M. bovis* infections, approximately one-third of *M. bovis*-infected cats remained negative to this antigen. Therefore, a cat generating a PPDB-biased, ESAT-6/CFP-10 negative response can only be diagnosed as infected with a member of the MTBC without inferring either *M. bovis* or *M. microti*, as ESAT-6/CFP-10 negativity does not rule out *M. bovis* infection. Other supporting veterinary and epidemiological data, such as the geographic location of the cat and its dietary history (Gunn-Moore et al., 2011; O'Halloran et al., 2019), can be used to assign greater or lesser suspicion to either one of these pathogens.

Non-tuberculous mycobacteria also cause a substantial burden of morbidity in cats (Baral et al., 2006; Gunn-Moore et al., 2011; Malik et al., 2013), and these infections can be just as devastating as cases of TB (Gunn-Moore, 2014). It is important to identify which species of mycobacteria is present as it is established from human medicine that treatment protocols and prognosis can differ between organisms (Haworth et al., 2017). Despite the small number of NTM-positive cats in this study, all gave a positive IGRA result compared to previous studies where approximately half of NTM-infected cats were IGRA positive (Rhodes et al., 2008a, 2011). Studies in humans have investigated the use of *M. avium*-sensitin to improve sensitivity for diagnosing *M. avium*-complex infections (Ra et al., 2011), but IGRAs typically perform poorly for diagnosing NTM infections (Lein et al., 1999; Ra et al., 2011). It has also been shown that NTM infections in humans can give false-positive results on IGRA testing for TB (Hermansen et al., 2014); typically these cross-reactive NTM, which includes *M. kansasii* (Sato et al., 2016), are RD-1-positive (Guinn et al., 2004; Arend et al., 2005; van Ingen et al., 2009). These RD-1 orthologues have been shown to generate a quantifiable T-cell response in cattle, but this immune response is less potent than for *M. bovis* (Vordermeier et al., 2007). One cat with *M. kansasii* (which has been described previously (Černá et al., 2020)) gave a positive ESAT-6/CFP-10 result using the new test cut-offs. One recommendation from this study would be to follow-up a PPDA-biased IGRA result with PCR and/or culture to confirm which NTM is present.

Exposure to environmental mycobacteria or mycobacterial co-infection can impair the ability to accurately diagnose MTBC infections (Hope et al., 2005; Jenkins et al., 2018). One cat in this study was diagnosed by PCR with *M. chelonae*, however, the IGRA result was consistent with *M. bovis* infection. Cross-reactivity from human *M. abscessus-chelonae* infections with the IGRA, where only TB antigens are detected (including ESAT-6 and CFP-10), has been recorded (Siddiqi et al., 2012; Hermansen et al., 2014). Unlike the human IGRA, the feline IGRA incorporates a comparative PPD response, which adds confidence for a MTBC infection. It is possible this cat had a dual *M. bovis-M. chelonae* infection, but as *M. chelonae* is a rapid growing mycobacterial species, and would therefore outgrow MTBC, only *M. chelonae* was detected on PCR (Simmon et al., 2011). This discordant result between IGRA and PCR reflects the complex pattern of naturally occurring mycobacterial infections in cats.

Despite previous studies suggesting excellent sensitivity of the IGRA for feline TB, approaching 100 % (Rhodes et al., 2011), this study which encompassed larger cat numbers identified a small number of cats with TB that tested negative for all mycobacterial antigens. These cats may have been tested early during infection and had not yet mounted a substantial immune response (Klenerman et al., 2002), therefore giving an IGRA negative result. In addition, advanced age has been consistently identified as a risk factor for false-negative IGRA results in humans with active TB (De Visser et al., 2015; Yamasue et al., 2020); the number of false-negative IGRA results in this study was too small to investigate further, but age was not identified as a risk factor for failure of PBMCs to respond appropriately to the positive mitogen control. As the IGRA measures IFNγ production by PBMCs, compartmentalisation of antigen-specific T-cells to the site of infection could result in false-negative IGRA results (Losi et al., 2007; Jafari et al., 2008). In the current study, other than one cat with localised cutaneous lesions, systemic disease was identified in all cats that gave a false-negative IGRA response. Given the disseminated distribution of lesions one may expect to identify circulating antigen-specific T-cells; however, there may be compartmentalisation of these antigen-specific T-cells to sites of disease, therefore resulting in reduced circulating populations of these cells.

Alternatively, there may be differences in PBMC populations to explain false-negative IGRA results. For example, expansion of $CD4^{+}CD25^{high}$ T-regulatory cells has been shown to inhibit BCG-specific production and release of IFNγ from $CD4^{+}$ in humans with active TB (Li et al., 2007). Further characterisation of the PBMC population in cats with false-negative results would help explore this theory further. Currently, in cases where the IGRA is negative but there is evidence of mycobacteria *i.e.* Ziehl-Neelsen (ZN) positive staining on tissue biopsy or cytology samples, a diagnosis of "non-*M. avium*, environmental mycobacteriosis" is ascribed; however, the results herein suggest that cats with systemic TB can also give negative IGRA results. The implications for misdiagnosis could unknowingly expose immunosuppressed individuals to a higher risk zoonotic pathogen and appropriate hygiene precautions may not be followed (Human Animal Infections and Risk Surveillance (HAIRS) group, 2014; O'Connor et al., 2019), or an ineffective treatment protocol could be instigated (Gunn-Moore, 2014). Therefore, an IGRA negative, ZN positive result in a cat should be followed-up with culture and/or PCR as appropriate, or repeat IGRA undertaken if the initial test was performed prior to the cat generating a substantial T-cell response.

Unlike in cattle, there is no statutory TB surveillance screening for cats. This, coupled with the fact that the IGRA is typically performed on cats where mycobacteriosis is suspected, means very few animals that do not have mycobacterial disease are tested. A total of 37 cats were identified that were diagnosed with a condition other than mycobacterial disease; to calculate test specificity this was taken as a proxy negative control cat group. Ideally a larger group of negative control animals would have been used to calculate test specificity. The OIE Test Validation guide (World Organisation for Animal Health (OIE), 2018) suggests for a specificity of 97–99 % to use up to 500 individuals for a 2% error of specificity estimate, and up to 80 individuals for a 5% error of specificity estimate respectively. Notwithstanding our lower-than-ideal control group numbers, our results suggest the IGRA

has excellent specificity for MTBC infections. Two negative control cats gave a PPDA-biased result, although both were ultimately diagnosed with a non-mycobacterial disease. It was theorised these results were due to exposure to NTM (Hope et al., 2005; Jenkins et al., 2018), either through hunting or from environmental sources (Gunn-Moore et al., 2011; Gunn-Moore, 2014). Positivity to ESAT-6/CFP-10 was recorded in one cat diagnosed with intestinal lymphoma; similarly, this cat had outdoor access and lived in a part of the UK with endemic *M. bovis* infection in wildlife and cattle, therefore it could have been exposed to *M. bovis* (or potentially RD-1 positive NTM) from the environment. Any IGRA positive result should be carefully interpreted in the light of the clinical signs, results of other diagnostics *i.e.* histopathology, and supporting epidemiological data to determine whether the IGRA result is of significance or whether the clinical signs are attributable to another disease condition.

To make meaningful interpretations of any diagnostic method, the test needs to perform appropriately. This study showed the highest proportion of test failures was due to insufficient IFNγ production by PBMCs stimulated by the positive mitogen control stimulus (PMA-Ca). Cattle IGRA data generally will show positive sample QC failure when blood samples are exposed to extremes of temperature during transit to the laboratory, reflecting a reduced viability of the sample. Cattle blood samples are therefore conveyed to the testing laboratory in temperature-controlled delivery boxes. In the current study, more PC failures were identified in spring and summer compared to autumn. Samples becoming too chilled, or too warm, could result in disruption to T-lymphocytes and reduced stimulation of these cells on subsequent co-incubation with mycobacterial antigens (Rothel et al., 1992). Use of thermoregulatory packaging materials to ship samples could help preserve the viability of T-lymphocytes, as temperature-controlled delivery is currently not used for cat IGRA samples. Also, it cannot be ruled out that the mitogen positive sample control might not be optimal for felines. However, previous studies have shown difficulties in effectively stimulating feline PBMCs with various other mitogen options (Rhodes et al., 2008a); and the use of PMA-Ca in domestic cats is supported by the development of a lion-specific IGRA which also uses PMA-Ca (Maas et al., 2012). Concanavalin A has been used as a mitogen in feline IGRA tests for *Toxoplasma gondii* and *Leishmania infantum* (Yin et al., 2015; Priolo et al., 2019), however, our previous experience was of poor or inconsistent IFNγ release following stimulation with this mitogen (S.R., unpublished data). Exploration of current thresholds for PMA-Ca showed they were statistically valid, although the criteria for a test to pass can be extended for those where the $OD_{PC}$ exceeds the $OD_{NC}$ by $\geq$ 0.20. Additional studies to identify mitogens or stimulation conditions that more reproducibly induce IFNγ may be warranted. For example, interleukin (IL)-12 has been shown to potentiate IFNγ release from stimulated T-lymphocytes (Gerosa et al., 1996; Donnell et al., 1999); co-incubating feline PBMCs with IL-12 may boost the IFNγ response in the IGRA. Failure to respond to the mitogen control and all mycobacterial antigens could also indicate a state of anergy (Schwartz, 2003; Brock et al., 2006; Chappert and Schwartz, 2010). Any test with a QC fail should be considered for retesting with a fresh sample, as well as potentially investigating for any causes of T-cell subset depletion or anergy in individual cases where additional information might suggest this is warranted.

Multivariate analysis also identified DxH (domestic short, medium, or longhair) cats as being more likely to have a PC failure compared to non-DxH cats. This could reflect the greater number of DxH cats compared to non-DxH cats, rather than an underlying immune dysfunction in DxH cats, given that non-DxH cats are more commonly associated with increased susceptibility or are over-represented for infectious diseases or immune disorders (Baral et al., 2006; Gunn-Moore et al., 2008; Golovko et al., 2013). Interestingly, in contrast to what has been observed in cattle (Rothel et al., 1992; Gormley et al., 2004), the duration from taking a blood sample to processing it testing was not associated with test failure. Regardless, it is still advisable to take samples no earlier than 48 h before the IGRA is to be performed to maximise test performance. There may have been other factors not captured by this study that could have contributed to PC failures, such as the cat being stressed at the time of sampling, as stress has been shown to inhibit IFNγ production in humans (Calcagni and Elenkov, 2006), or rarer causes such as deficiencies in leucocyte integrins, resulting in reduced T-cell proliferation (Bauer et al., 2017). A small number of cats produced excessive IFNγ in the NC wells. Natural Killer (NK) cells have been identified as a cause of false-positive IGRA results in young cattle (Olsen et al., 2005), and these cells are a major source of IFNγ production early in infections (Bancroft, 1993). However, given the small number of tests where excessive IFNγ was observed in the NC, it seems that the contribution of NK cells to IGRA test failures in cats is minimal. Similarly, a small number of tests failed due to excessive variation between replicates; if this occurs, the ELISA should be repeated to improve confidence in the results (Tuuminen et al., 2010).

There are some limitations of this study. While PCR diagnostics for mycobacterial infections are well established, false-positive (Noordhoek et al., 1994) and false-negative (Nguyen et al., 1996) results have been reported, as well as misclassification of NTM as MTBC infections (Chedore et al., 2006; Rodríguez-Aranda et al., 2010). Mycobacterial PCR methodologies can vary widely; three of the PCR tests used by commercial diagnostic laboratories in this study could not discriminate between members of the MTBC, which is important in cases of feline mycobacteriosis due to the different zoonotic risk posed by *M. bovis* compared to *M. microti*. In this study 21 cats had a PCR diagnosis of MTBC infection, but the exact species could not be identified. Where PCR methodologies could discriminate between members of the MTBC, this was dependent on sufficient DNA being extracted (which may have been a limiting factor where formalin-fixed paraffin-embedded tissue samples were tested) and whether owner finances could permit further testing. Ideally culture, PCR and IGRA would be undertaken in all cases; this could allow a Bayesian approach to determine test parameters (Dendukuri and Joseph, 2001), which is recommended in cases where a 'gold standard' diagnostic test does not exist. While specialist mycobacterial culture is the reference standard diagnostic test for feline mycobacterial infections in Great Britain (Middlemiss and Clark, 2018), it may not be considered 'gold standard' due to its poor sensitivity, the length of time required for mycobacteria to grow, the risk of contamination with environmental organisms, and the difficulties accessing representative tissues for submission especially where there is only limited disease, or where invasive surgery is needed to collect samples. The IGRA also has limitations, one being the presence of co-morbidities such as retroviral infections which can reduce the number of $CD4^+$ cells, which could potentially affect the performance of the test (Aabye et al., 2009). However, FeLV and/or FIV infections are uncommon in cats with mycobacterial disease in Great Britain (Gunn-Moore et al., 2011) and out of 33 cats with mycobacteriosis that were tested for retroviruses, only one was FIV-positive. Despite this, over half of the cats with culture or PCR-confirmed mycobacteriosis were not tested for retroviruses and there may be other unidentified causes of immunodeficiency that may affect the performance of the IGRA. A prospective study should seek to recruit cats undergoing complete investigation *e.g.* thoracic and abdominal imaging, retrovirus screening and determination of serum total and ionised calcium concentrations.

Finally, the proposed new cut-offs were applied to the same set of cats from which these values were derived. The external validity of these thresholds should be determined to assess whether the adjusted cut-off values are appropriate for an alternative population (Steckler and McLeroy, 2008). If these new thresholds show better sensitivity and specificity compared to current cut-off values, this will provide further support to adopting these adjustments. Mycobacterial infections, particularly with MTBC pathogens, are identified with relative frequency in cats in Great Britain (Gunn-Moore et al., 2011, 2013), and as the IGRA is used in a population of cats where mycobacteriosis is suspected, this results in a high positive predictive value (PPV), whereas the

negative predictive value (NPV) is reduced. If the IGRA was used for screening a population where mycobacterial infection is not expected, the lower prevalence would decrease the PPV, however, the NPV would improve. This could have important implications for animal and human health in both directions *e.g.* a negative result in a cat where infection is highly suspected may result in that animal not being treated, compromising welfare, whereas a positive result in a cat with low prevalence of disease could result in that animal being treated unnecessarily, or potentially euthanised. An additional factor is differences in the prevalence of species of mycobacteria infecting cats; while MTBC infections are found throughout Great Britain, the distribution of *M. bovis* and *M. microti* differ (Gunn-Moore et al., 2011), so a lower ESAT-6/CFP-10 threshold may be advisable for cats being tested from regions of Great Britain where *M. bovis* is present to increase antigen sensitivity and improve the PPV. Antigen cut-off thresholds may also require further manipulation to maximise test performance in regions where NTM infections are more prevalent, either within Great Britain *e.g.* Eastern England (Gunn-Moore et al., 2011), or further afield such as Australia (Malik et al., 2000).

In conclusion, the data presented supports the statistical adjusting of current cut-off values for the IGRA to provide a more tailored 'feline-specific' test. Data presented in this report suggests a resulting improved test performance for the diagnosis of mycobacterial infections in cats. The IGRA has good sensitivity for MTBC infections, with acceptable scope to discern between *M. bovis* and *M. microti* infection. For NTM, while the IGRA is reasonably sensitive, it is limited in that it cannot discriminate between different mycobacterial species. In cases where the IGRA result is close to test thresholds, or results do not conform to what is expected, a degree of flexibility and consultant discretion should be applied, provided the interpretation is supported by clinical and epidemiological data.

## 5. Addendum

The authors have since recommended follow-up culture or PCR testing in cases with a PPDA-biased IGRA result. A positive PCR result was obtained for nine cats: five had MTBC infections and four had NTM. Of the MTBC infections, two were identified as *M. microti*; the species was not determined in the remaining three cats due to insufficient sample for further testing (two cats) or financial restrictions (one cat). The NTM cases comprised two cats diagnosed with *M. lepraemurium* (including one that was positive to ESAT-6/CFP-10), one with *M. malmoense* and one with *M. smegmatis*.

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## Declaration of Competing Interest

The authors report no declarations of interest.

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# Serial Interferon-Gamma Release Assay (IGRA) Testing to Monitor Treatment Responses in Cases of Feline Mycobacteriosis

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**Abstract:** The interferon-gamma release assay (IGRA) is used to diagnose cases of feline mycobacteriosis, but the use of serial testing to monitor treatment responses has not been evaluated in this species. From a population of cats that underwent IGRA testing for diagnostic investigation, individuals were identified with a pre- and end-of-treatment IGRA that passed control thresholds. The number of cats which reverted to negative at the end-of-treatment IGRA, changes in paired antigen-specific optical density (OD) values and differences in the pre-treatment antigen-specific OD values for those which underwent reversion were compared. Factors to explain reversion or recurrence of disease post-treatment were explored. Four of 18 cats (22%) reverted to negativity at the point of clinical resolution ($p = 0.33$), there was no difference in paired antigen-specific OD values ($p \geq 0.12$), and cats that reverted did not have a lower baseline OD value ($p = 0.63$). No statistically significant factors were identified to predict reversion ($p \geq 0.08$). Remaining positive at the end of treatment IGRA was not associated with recurrence of disease post-treatment ($p = 0.34$). Overall, these data suggest there is limited value in the use of the IGRA to monitor treatment responses in cats.

**Keywords:** cat; tuberculosis; mycobacteria; monitoring; interferon-gamma release assay; diagnostics



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## 1. Introduction

Mycobacteriosis is a substantial issue within the domestic cat population in Great Britain, with an estimated 1% of all routine feline biopsy submissions showing changes suggestive of mycobacterial disease [1]; however, many cases may not initially be recognised by practitioners. There are many challenges when faced with a suspected case of mycobacterial disease, notably obtaining an accurate diagnosis, monitoring the response to therapy, and identifying when treatment with antimicrobials can be stopped. Current options for diagnosing mycobacterial infections in cats are limited to specialist mycobacterial culture, molecular-based methods, i.e., polymerase chain reaction (PCR) and genome sequencing, or the interferon-gamma (IFNγ) release assay (IGRA) [2].

Initially established for diagnosing tuberculosis (TB) in cattle where tuberculin skin testing failed to identify all infected animals [3], the IGRA has since been adapted for the diagnosis of TB in other species [4,5], including cats [6]. These assays detect the

cellular immune response through secretion of IFNγ by T-cells following stimulation with mycobacterial antigens [7]. The feline IGRA is designed to detect and discriminate between infection with the two most common causes of mycobacteriosis in this species in Great Britain, i.e., *Mycobacterium* (*M.*) *microti* and *M. bovis* [8], which both cause TB. The IGRA also has some capacity to detect infections with *M. avium* and other non-tuberculous mycobacteria [9]. While the IGRA appears useful for diagnosing mycobacteriosis in cats, its clinical utility for monitoring the response to treatment in this species is unknown.

The IGRA is an immunological test based on sensitised antigen-specific T-cells responding to repeated antigenic stimulation in vitro [7], and its methodologies and use in people have been thoroughly evaluated [10]. It is thought that the number of IFNγ-secreting effector T-cells is proportional to the antigenic load, which is taken to reflect the bacterial load [11,12], and that successful antimycobacterial treatment will result in a decrease of this antigenic stimulus and a subsequent reduction in the number of antigenic-specific effector T-cells [13]. Therefore, it has been proposed that serial IGRA testing for those receiving antimycobacterial chemotherapy may be of some benefit [14]. Early investigations of serial IFNγ responses showed that region of difference-1 (RD-1) markers, such as early secreted antigenic target 6kDa (ESAT-6), were more likely to decrease in those with a good response to treatment than responses to the crude purified protein derivative (PPD) antigen [15], and human IGRA tests have since been designed using RD-1 antigens. Despite this, in people, reversion from IGRA positivity at the time of diagnosis to negativity at the conclusion of therapy has been shown to range from as low as 5.5% [16] up to 71% [17]. However, remaining positive at the end of treatment IGRA is not always associated with an unfavourable outcome [16,18]. While changes in the categorical classification of IGRA results has not been suggested as helpful for assessing successful response to therapy, the magnitude of change in the IFNγ response may be of more benefit, as a small majority of studies has shown a significant decrease in this measure between the start and end of treatment [19]. Despite this, some studies have shown no change between pre- and post-treatment IFNγ levels [20,21], while others have shown an increase [18]. There are limitations to some of the methodologies of these studies making it difficult to accurately compare and collate findings, namely, variable sample sizes, genetic differences across populations studied, the level of TB exposure across different populations, co-morbidities, and the censoring of data exceeding the upper limit of quantification. A systematic review of 30 studies using serial IGRA testing to monitor the response to anti-TB chemotherapy failed to show a consensus in the rate of reversion with highly variable results between patients within studies, as well as rates across studies. Therefore, repeat IGRA testing is not recommended to monitor individual patient responses to treatment in humans [19].

Serial IGRA testing has been previously reported in a small number of cats treated for confirmed or suspected mycobacteriosis [9,22], most of whom remained positive despite apparent resolution of disease. However, it is unknown whether these findings are representative of the IFNγ response in a larger population of cats or whether we should aim for reversion to IGRA negativity when treating cases of feline mycobacterial disease.

The objective of this study was to determine whether serial IGRA tests are of benefit for monitoring the response to antimycobacterial therapy in cats. We assessed whether reversion to IGRA negativity was observed in association with apparent clinical resolution. We also measured the change in the magnitude of the IFNγ response and investigated factors that might influence reversion to IGRA negativity or the risk of recurrence of mycobacterial disease post-treatment.

## 2. Results

### *2.1. Study Population Selection and Summary Statistics*

The selection process to identify eligible cats that underwent serial IGRA testing while receiving antimycobacterial treatment is shown in Figure 1. In total, 741 IGRAs were performed on 594 individual cats, with 107 cats having had more than one test. Fifty-one cats had at least two tests which passed the test criteria (see *4.1. Study Population Selection*).

Of the 56 cats that did not pass these criteria: 49 were excluded as they did not meet the conditions for a passing optical density (OD) positive control (PC) and/or positive control–negative control (NC) OD values, four had coefficient of variation (CV) values exceeding 30% for at least one test condition (including six that also did not meet the $OD_{PC}$/$OD_{PC}$-$OD_{NC}$ thresholds), and three had average $OD_{NC}$ values greater than 0.30. Active mycobacterial disease was diagnosed in 36 of the 51 cats with at least two tests meeting the IGRA inclusion criteria, and of these 18 were included for final analysis as they did not have a previous history of being treated for mycobacterial disease. Both tests passed quality control (QC) thresholds and they were conducted (pre-treatment and at the end of treatment) within the same episode of active clinical disease.

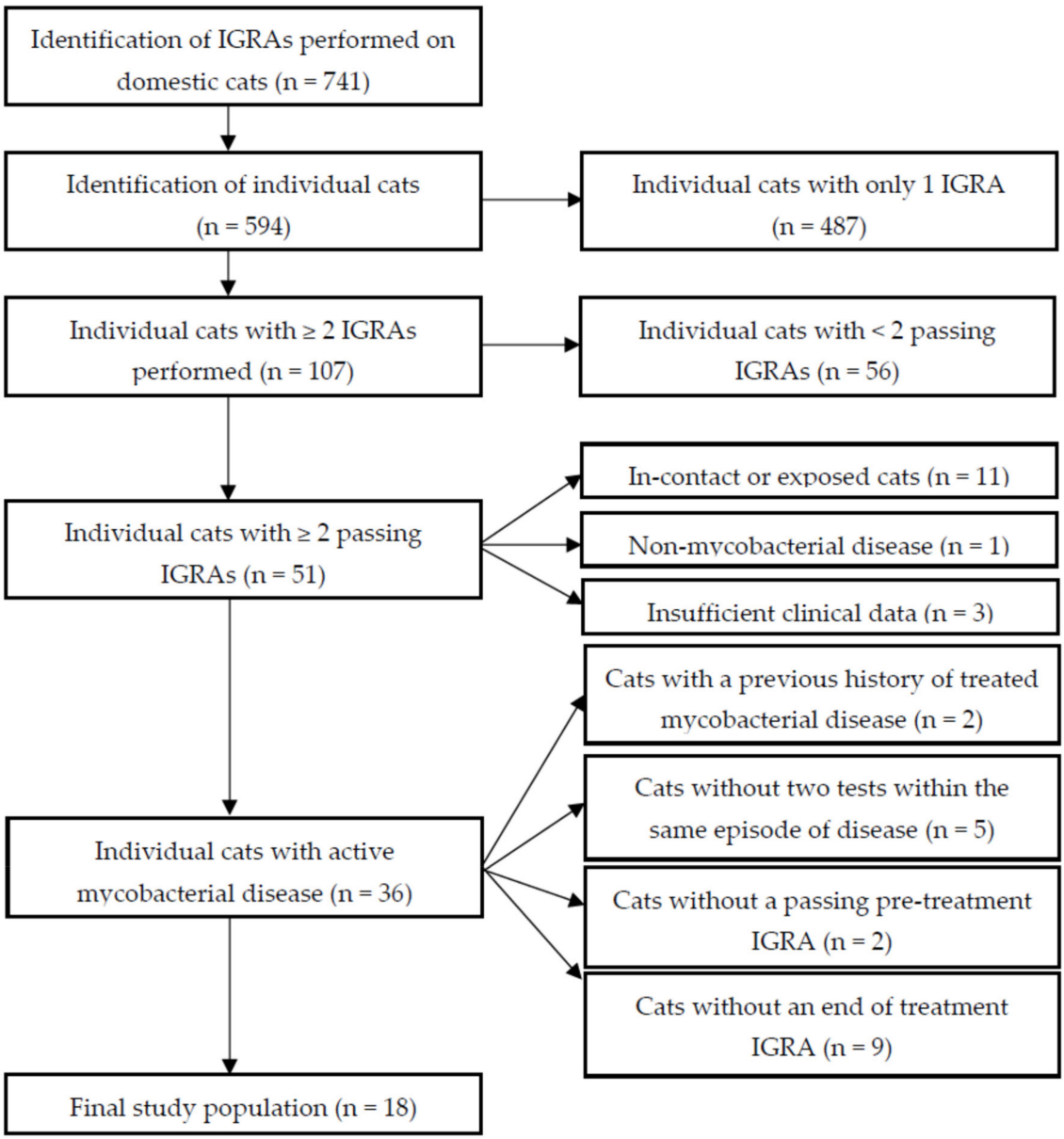


**Figure 1.** Flow diagram to show the decision-making process for identifying cats assessed by serial interferon-gamma release assay (IGRA) for subsequent analysis. IGRA = interferon-gamma release assay; Passing = optical density (OD) positive control (PC) $\geq$ 0.40, OD negative control (NC) $\leq$ 0.30, and $OD_{PC}$-$OD_{NC}$ $\geq$ 0.10, or if $OD_{PC}$ < 0.40, $OD_{PC}$-$OD_{NC}$ $\geq$ 0.20. Coefficient of variation between duplicate PC and NC wells $\leq$ 30%.

Clinical data for the cats in this study are summarised in Table 1. The median age of cats at the time of the pre-treatment IGRA was 6.75 years (0.5–13 years), and 15 (83%) were

neutered males. Twelve cats (67%) were domestic short-hair (DSH); the remaining breeds were Siamese (n = 4), Bengal (n = 1), and Tonkinese (n = 1). Three cats were diagnosed with *M. bovis* by culture, one cat was culture- and PCR-positive for *M. microti,* and three further cats were diagnosed with *M. tuberculosis*-complex (MTBC) infection on PCR [23], but there was insufficient DNA to define the infectious agent further. Two cats were negative for *Mycobacterium* species on culture, and another cat was culture- and PCR-negative. The remaining eight cats did not undergo culture or PCR testing. Lymphadenopathy was identified in 13 cats (72%), including one cat with tonsillar lymphoid hyperplasia, followed by cutaneous lesions in seven (39%) and ocular lesions in three (17%). Twelve cats (67%) had an abnormal pulmonic lung pattern on radiography or computed tomography (CT). Testing for feline leukaemia virus antigen and feline immunodeficiency virus antibody was performed in five cats and all were negative. Hypercalcaemia was identified in four of nine cats tested (44%); one cat had increased total calcium, but ionised calcium was within the reference interval, another cat with increased total calcium did not have ionised calcium measured, and the remaining two cats both had increased ionised calcium.

Therapeutic interventions varied. The median duration of antimycobacterial treatment was six months (range 3–24 months). Ten cats (56%) were maintained on 'triple therapy' with rifampicin, azithromycin, or clarithromycin, and pradofloxacin for the entire duration of their treatment protocol; two of these cats also underwent surgery (i.e., enucleation) prior to medical management. Four cats (22%) were started on 'triple therapy', with discontinuation of rifampicin after two and six months in two cats, whereas azithromycin was discontinued in two cats after two and three months. Azithromycin or clarithromycin were substituted for erythromycin in one cat, in conjunction with rifampicin and pradofloxacin. One cat was treated with rifampicin, azithromycin, and marbofloxacin, with discontinuation of rifampicin after three months. A protocol of rifampicin, pradofloxacin, and clindamycin was started in one cat with suspected concurrent toxoplasmosis; clindamycin was then replaced with azithromycin after three months. The final cat was treated with a combination of erythromycin, doxycycline, and pradofloxacin.

Recurrence of clinical signs attributable to mycobacteriosis following resolution of disease was identified in five cases, all of which had radiographic evidence of pulmonic disease. The median duration to the onset of recurrent clinical signs was 16 months (3–27 months). Two of these cats were subsequently euthanased, whereas treatment was re-instated for the other three cats; clinical resolution was achieved in two of these cats, while the third was lost to follow-up.

### *2.2. Qualitative Classification of IGRA Results*

Pre- and end-of-treatment IGRA results were interpreted according to the pattern of responses to each antigen and scored on a positive–negative basis (Table 2). All 18 cats were positive on the pre-treatment IGRA, showing a biased response to PPD from *M. bovis* (PPDB), suggesting infection with a member of the MTBC. Three cases were positive to early secreted antigenic target 6kDa-culture filtrate protein 10kDa (ESAT-6/CFP-10 [E6C10]); two were culture-positive for *M. bovis* (cases 9 and 11). One cat (case 2) had a culture-confirmed diagnosis of *M. bovis* infection, but was negative to E6C10 on IGRA. At the end-of-treatment IGRA, 14 (78%) were persistently positive and four (22%) underwent reversion to negativity. A PPDB-biased response was maintained in all 14 persistently positive cats. In addition to the two E6C10-positive cats with culture-confirmed *M. bovis* infection on the initial IGRA, two further cats were now positive to E6C10 at the end-of-treatment IGRA. Neither of these cats were case 2, the *M. bovis* culture-positive cat, which remained negative to E6C10. The proportion of cats that reverted to a negative end-of-treatment IGRA was not statistically different to that seen in humans treated for active TB ($p = 0.33$), i.e., the rates of reversion are similar.

**Table 1.** Summary of the details of the cats included in this study.

| Case | Age (Years) | Gender | Breed | Culture/PCR | FeLV Ag/FIV Ab | Serum Calcium Concentration | Clinical Disease | Treatment & Duration (Months) | Outcome & Follow-up Duration (Months) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 7 | MN | DSH | MTBC [a] | NP | NP | Retrobulbar ocular mass, submandibular lymphadenopathy | R/A/P (3); surgery | Resolved (9) |
| 2 | 3 | FN | Siamese | *M. bovis* [b] | NP | NP | Lip mass, submandibular lymphadenopathy, bronchointerstitial lung pattern [c] | R/A/P (6) | Resolved (18) |
| 3 | 0.5 | MN | DSH | MTBC [a] | NP | Normal (total) | Interstitial lung pattern [c] | R/A/P (3); R/P (15) | Resolved (3) |
| 4 | 8.5 | MN | DSH | NP | Negative | Increased (total) Normal (ionised) | Nasal mass, submandibular lymphadenopathy | R/A/P (6) | Resolved (48) |
| 5 | 7 | MN | Siamese | *M. microti* [a,b] | Negative | Normal (total) | Multiple cutaneous masses, bronchointerstitial lung pattern [c] | R/A/P (2); R/P (6) | Resolved (12) |
| 6 | 7 | MN | DSH | NP | NP | NP | Bilateral submandibular lymphadenopathy, bronchointerstitial lung pattern [c] | R/A/P (7) | Recurrence of submandibular lymphadenopathy 27 months later; retreated with R/A/P, lost to follow-up |
| 7 | 6 | MN | Siamese | MTBC [a] | NP | NP | Submandibular mass, submandibular lymphadenopathy, bronchial lung pattern [c] | R/A/P (3) | Re-presented with mass on lip 17 months later; retreated with R/A/P for four months, resolved |
| 8 | 11 | MN | Siamese | NP | Negative | Increased (ionised) | Tonsillar lymphoid hyperplasia, alveolar lung pattern [c] | R/A/M (3); A/M (12) | Resolved (12) |
| 9 | 7 | MN | DSH | *M. bovis* [b] | NP | NP | Conjunctival mass | R/A/P (3); A/P (2) | Resolved (6) |
| 10 | 6.5 | FN | DSH | Negative [b] | NP | NP | Multiple cutaneous masses, peripheral lymphadenopathy bronchointerstitial lung pattern [c] | R/A/P (8) | Recurrence of cutaneous masses and peripheral lymphadenopathy eight months later; euthanased |

**Table 1.** *Cont.*

| Case | Age (Years) | Gender | Breed | Culture/PCR | FeLV Ag/FIV Ab | Serum Calcium Concentration | Clinical Disease | Treatment & Duration (Months) | Outcome & Follow-up Duration (Months) |
|---|---|---|---|---|---|---|---|---|---|
| 11 | 5 | MN | DSH | *M. bovis* [b] | NP | Normal (total) | Discharging cutaneous mass, perihilar lymphadenopathy, interstitial lung pattern [c] | R/A/P (6); A/P (18) | Resolved (42) |
| 12 | 13 | MN | Bengal | NP | NP | Normal (total) | Generalised lymphadenopathy, bronchointerstitial lung pattern [d] | R/A/P (9) | Recurrence of clinical signs three months later; euthanased |
| 13 | 5.5 | MN | DSH | Negative [b] | NP | NP | Generalised lymphadenopathy, bronchointerstitial lung pattern [c] | R/A/P (6) | Resolved (6) |
| 14 | 7.5 | MN | DSH | NP | Negative | NP | Multiple cutaneous masses, submandibular lymphadenopathy | R/P/Cd (3); R/A/P (3) | Resolved (36) |
| 15 | 3 | FN | DSH | NP | NP | NP | Submandibular lymphadenopathy | E/D/P (6) | Resolved (6) |
| 16 | 1.5 | MN | Tonkinese | NP | Negative | Increased (total, ionised) | Diffuse interstitial lung pattern [c] | R/Ct/P (8) | Recurrence of clinical signs 16 months later; retreated with R/Ct/P for eight months, resolved |
| 17 | 8.5 | MN | DSH | NP | NP | Normal (total) | Generalised lymphadenopathy | R/E/P (4) | Resolved (24) |
| 18 | 2 | MN | DSH | Negative [a,b] | NP | Increased (total) | Panuveitis, bronchointerstitial lung pattern [d] | R/A/P (3); surgery | Resolved (12) |

FeLV Ag = feline leukaemia virus antigen. FIV Ab = feline immunodeficiency virus antibody. MN = male neutered. FN = female neutered. DSH = domestic short-hair. MTBC = *Mycobacterium tuberculosis*-complex. NP = not performed. R = rifampicin. A = azithromycin. P = pradofloxacin. M = marbofloxacin. E = erythromycin. D = doxycycline. Ct = clarithromycin. Cd = clindamycin. [a] = PCR diagnosis. [b] = culture diagnosis. [c] = thoracic radiography. [d] = computed tomography.

**Table 2.** Binary classification and interpretation of interferon-gamma release assay (IGRA) results prior to starting antimycobacterial therapy and at the point of treatment cessation, and categorisation of the pattern. Positivity was ascribed to an IGRA with a positive result to any test antigen, whereas a negative result indicated an optical density value below positivity-thresholds for all test antigens.

| Case | Pre-Treatment IGRA | IGRA Result | End-of-Treatment IGRA | IGRA Result | Pattern |
|---|---|---|---|---|---|
| 1 | Positive | B > A | Positive | B > A | Persistent positive |
| 2 | Positive | B > A | Positive | B > A | Persistent positive |
| 3 | Positive | B > A | Positive | B > A, E positive | Persistent positive |
| 4 | Positive | B > A | Positive | B > A | Persistent positive |
| 5 | Positive | B > A | Positive | B > A | Persistent positive |
| 6 | Positive | B > A | Positive | B > A | Persistent positive |
| 7 | Positive | B > A | Positive | B > A | Persistent positive |
| 8 | Positive | B > A | Positive | B > A, E positive | Persistent positive |
| 9 | Positive | B > A, E positive | Positive | B > A, E positive | Persistent positive |
| 10 | Positive | B > A | Positive | B > A | Persistent positive |
| 11 | Positive | B > A, E positive | Positive | B > A, E positive | Persistent positive |
| 12 | Positive | B > A | Positive | B > A | Persistent positive |
| 13 | Positive | B > A | Positive | B > A | Persistent positive |
| 14 | Positive | B > A | Positive | B > A | Persistent positive |
| 15 | Positive | B > A | Negative | Negative | Reversion |
| 16 | Positive | B > A | Negative | Negative | Reversion |
| 17 | Positive | B > A | Negative | Negative | Reversion |
| 18 | Positive | B > A, E positive | Negative | Negative | Reversion |

Tuberculin-bias is indicated by >. B = purified protein derivative (PPD) from *M. bovis* (PPDB). A = PPD from *M. avium* (PPDA). E = early secreted antigenic target 6kDa/culture filtrate protein 10kDa (ESAT6/CFP10).

### 2.3. Quantitative Evaluation of IGRA Responses

To account for intra-cat variability between IGRA results that passed QC thresholds, paired $OD_{PC}$ values for each cat at pre- and end-of-treatment IGRA were compared to identify overlap in these values, allowing for comparison of antigen OD values. Eleven of the 18 cats had overlapping $OD_{PC}$ values between the pre- and end-of-treatment IGRA (data not shown).

Of these 11 cats, eight (73%) showed a decrease in the $OD_{PPDB}$ value between the pre- and end-of-treatment IGRA (Figure 2A). There was a decrease in the $OD_{E6C10}$ value in three of the five cats that were culture- or IGRA-positive for infection with *M. bovis* (Figure 2B). The median OD values for both PPDB and E6C10 decreased (0.49 to 0.22 $OD_{PPDB}$; 0.15 to 0.06 $OD_{E6C10}$), but there was no statistically significant difference between paired OD values for either antigen ($OD_{PPDB}$, $p = 0.12$; $OD_{E6C10}$, $p = 0.81$).

The pre-treatment $OD_{PPDB}$ values were compared between cats that were persistently positive at the end-of-treatment IGRA (n = 8) and those that reverted to negative (n = 3) (Figure 3). The median $OD_{PPDB}$ value was higher in the persistently positive group (0.58) compared to the reversion group (0.38), however this difference was not statistically significant ($p = 0.63$).

### 2.4. Logistic Regression Analysis

Logistic regression was performed to identify potential factors that may predict reversion to IGRA negativity at the end-of-treatment IGRA or recurrence of disease. For

reversion, a multivariate model with age, gender, breed (DSH vs non-DSH), the presence or absence of pulmonic disease, and treatment regimen ('triple therapy' as at least part of the treatment protocol for at least three months in cases without pulmonic disease and at least six months in cases with pulmonic disease vs 'triple therapy' as at least part of the treatment protocol for an insufficient duration of time as well as all other protocols) was constructed. The multivariate model then underwent stepwise multidirectional reduction to its simplest components. Exploration of the full multivariate model revealed high collinearity between non-DSH cats and the presence of pulmonic disease, with no statistically significant factors for IGRA reversion identified. The reduced model identified younger age and the absence of pulmonic disease as the most important factors in predicting reversion to IGRA negativity at the end of treatment, but neither were statistically significant (age, $p = 0.08$, odds ratio [OR] = 0.50 [0.23−1.09]; pulmonic disease, $p = 0.17$, OR = 0.05 [0.0006–3.88]).

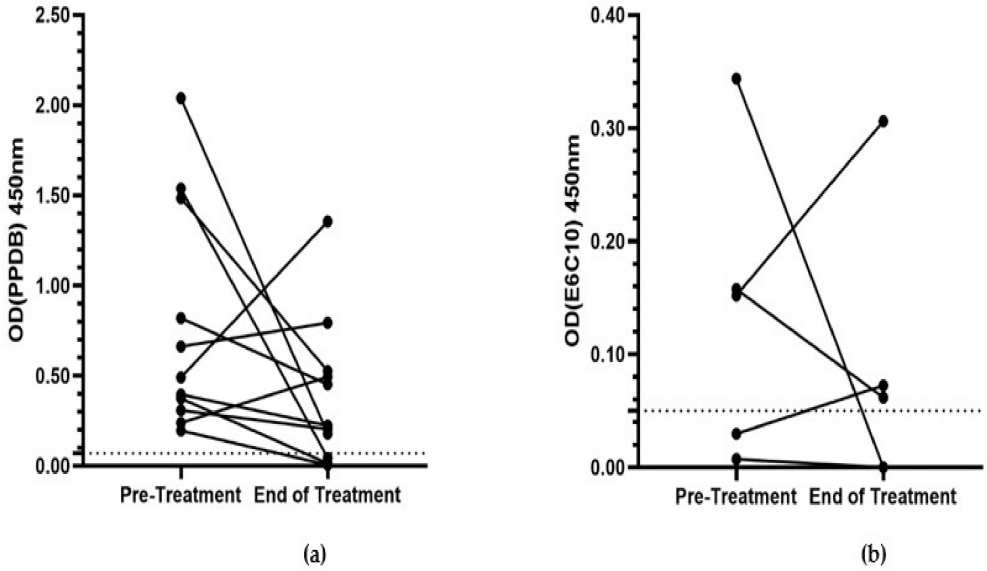


**Figure 2.** Paired average negative control corrected-OD values for pre- and end-of-treatment IGRA for (**a**) PPDB (n = 11) and (**b**) E6C10 (n = 5). The dotted line at OD 0.07 signifies the threshold for PPDB positivity. The dotted line at OD 0.05 signifies the threshold for E6C10 positivity. OD = optical density. IGRA = interferon-gamma release assay. PPDB = purified protein derivative from *M. bovis*. E6C10 = ESAT-6/CFP-10 antigenic cocktail.

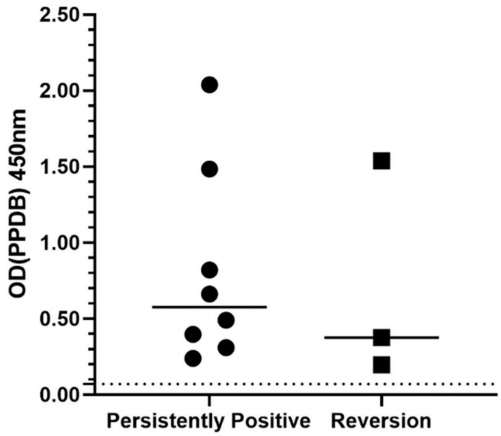


**Figure 3.** Pre-treatment IGRA average $OD_{PPDB}$ values for cats who remained positive at the end-of-treatment IGRA (n = 8) compared to those which reverted to negative (n = 3). The solid line shows the median OD value for each group. The dotted line at OD 0.07 signifies the threshold for PPDB positivity. OD = optical density. IGRA = interferon-gamma release assay. PPDB = purified protein derivative from *M. bovis*.

Factors included in the multivariate model for the risk of disease recurrence included those modelled for reversion, in addition to remaining persistently positive at the end-of-treatment IGRA. There was no statistically significant association between remaining persistently positive at the end-of-treatment IGRA and recurrence of disease ($p$ = 0.34, OR = 0.06 [0.0002–19.26]). Quasi-complete separation of the data was identified; radiographic evidence of pulmonic disease at the initial presentation was recorded in all five cats that had recurrence of clinical signs attributable to mycobacterial disease, compared to seven cats (54%) that did not redevelop clinical signs of mycobacteriosis. Pulmonic disease was the only factor remaining in the model following multidirectional stepwise reduction.

## 3. Discussion

This study investigated serial IGRA testing in cats with active mycobacterial disease and whether it can be used to monitor the response to antimycobacterial treatment. Fourteen out of 18 (78%) cats were persistently positive across the pre- and end-of-treatment IGRA, despite apparent clinical resolution of disease. Where comparable, paired OD values for PPDB and E6C10 antigen responses showed no statistically significant difference between the pre- and end-of-treatment IGRA, although the median OD value for both antigens was slightly lower at the end-of-treatment IGRA compared to the pre-treatment results. There was also no statistically significant difference between the pre-treatment $OD_{PPDB}$ value for cats that remained persistently positive to those that reverted to negative at the end-of-treatment IGRA. No statistically significant factors were identified to predict reversion to negativity at the end-of-treatment IGRA, and there was no association between the end-of-treatment IGRA result and recurrence of disease post-treatment. However, all five cats that had recurrence of clinical signs attributable to mycobacteriosis had evidence of pulmonic disease as part of their initial diagnostic investigation, compared to similar changes being identified in only seven of 13 cats (54%) that did not have recurrence of clinical signs during the available period of follow-up.

In line with previous studies on mycobacterial infections in Great Britain [8], most cases reported here were neutered male cats. The most common breed was the DSH, which reflects the domestic cat population in Great Britain, and the median age was nearly seven years old, although disease was identified in cats as young as six months old. While only seven cats presented with cutaneous lesions, 13 had a lymphadenopathy. Typically, cases of feline mycobacterial disease present with cutaneous nodules, which may be ulcerating and/or have discharging sinus tracts, with a secondary lymphadenopathy [8,24]. It may be the case that small skin lesions were not identified in some of the cats in this study, or subcutaneous masses closely associated with peripheral lymph nodes (i.e., submandibular and popliteal lymph nodes) may have not been identified as unique entities, or these cases presented at a later stage where lymph node changes were more prominent. An abnormal radiographic lung pattern was present in two-thirds of the cats in this study (12 of 18), with a bronchial/interstitial pattern most reported, which is in keeping with previous studies [25,26]. This is putatively due to haematogenous spread of bacteria from the primary site of infection, rather than inhalation of aerosolised mycobacteria which would more likely result in cavitary lesions. Clinical resolution of disease with no evidence of recurrence of clinical signs attributable to mycobacterial disease during the period of follow-up for each individual cat was achieved in 13 cats (72%), which is substantially higher than previously reported [27]. Other than one cat (case 15), all were treated with a combination of rifampicin, a macrolide, or azalide, and a fluoroquinolone for at least two months, although the choice of drugs and the duration varied greatly between cases. While further investigation is required, this would suggest the use of some combination of these drugs is suitable for treating cases of feline mycobacterial disease.

Other than in a small number of cases [9,22], the use of serial IGRA for monitoring the response to antimycobacterial treatment in cats has not been investigated. The data from the current study reflect observations from human studies of treating active mycobacterial disease, in that reversion to IGRA negativity at the end of antimycobacterial therapy is

uncommon [19]. Using feline-specific cut-off values to score IGRA antigen responses (Mitchell et al., manuscript in preparation), all cases included in this study were positive at the pre-treatment IGRA, and 78% of cases remained persistently positive at the end-of-treatment IGRA despite apparent clinical resolution. This rate of persistent positivity is similar to a pooled value of persistently positive humans following treatment for active TB ($p = 0.33$); of 982 patients, 660 remained persistently positive (67%) [19]. It has been suggested that maintenance of IGRA positivity could be due to improvement of the immune response in individuals undergoing treatment [28] or an increase in the proportion of IFNγ-secreting CD4+ T-cells [18]. It has also been recognised that clinically healthy individuals previously treated for tuberculosis can retain specific effector memory T-cell responses long after successful resolution of disease, although the precise mechanism driving this remains unknown [29].

Quantitative classification of the IGRA response in animals is less straightforward than in people, as these results are often reported as OD values [30,31]. Intra-subject variability with repeat IGRA testing is recognised, further complicating the ability to accurately compare serial IGRA results [28,32]. This variability can be problematic when interpreting borderline test results, possibly accounting for reversions in the absence of treatment, or conversions with no known exposure to *Mycobacterium* species [33]. In this study, a decrease in the median OD value was observed between the pre- and end-of-treatment IGRA for both PPDB and E6C10, although these differences were not statistically significant. Some cats showed an increase in the OD value between tests, despite apparent clinical resolution; this may be a result from continued expansion of T-cell subsets despite a decrease in the antigenic load [11]. Other studies have shown a decrease in the response to RD-1 antigens to be a better indicator of response to antimycobacterial therapy compared to PPD [15], but this was not observed in this study. This could be due to the small number of cats infected with mycobacteria that encode ESAT-6/CFP-10 in this study, and that approximately 20% of cats infected with *M. bovis* may not produce a substantial IFNγ response to the E6C10 cocktail [9].

It has been suggested that IGRA reversion is more likely in humans with a lower pre-treatment IFNγ concentration [13,34]; however, this observation is not consistent [17]. End-of-treatment IGRA reversion was identified in four cats in this study. While the pre-treatment median $OD_{PPDB}$ value for cats that reverted (where comparable data was available) was lower than those that remained positive, this difference was not statistically significant. This is probably influenced by the small study population. Logistic regression did not identify any factors associated with reversion to IGRA negativity; although not significant ($p = 0.08$), this study did show a slight trend with a decrease in the odds of reverting to a negative end-of-treatment IGRA with an increase in age (OR 0.50 [0.23–1.09]). This is similar to studies in humans, where older individuals were more likely to remain positive at the end-of-treatment IGRA despite resolution of disease [35]. One feature of ageing in humans is an increase in the number of memory T-cells; however, chronic antigenic stimulation can also result in the development of an oligoclonal population with impaired function [36]. While there are few studies describing changes in the feline immune system with age, it has been shown that older cats have reduced numbers of many cell populations, including CD4+ T-cells and CD56+ Natural Killer cells, as well as a reduction in the CD4:CD8 ratio [37,38]. Changes in immune cell populations and function with age may be reflected with this decrease in the odds of IGRA reversion with age.

In the current study, recurrence of disease was not associated with a persistently positive pattern of IGRA results, albeit the number of cats with recurrence of disease was low so the results should be interpreted cautiously. All cats who re-presented with clinical signs suggestive of mycobacterial disease had evidence of pulmonic disease on thoracic imaging when they initially presented. This may reflect a reduced capacity for the host immune system to restrict the spread of mycobacteria from the primary site of infection, or differences in the virulence of different species and/or strains of mycobacteria. Consequently, once treatment has been stopped on the basis of the cat

achieving clinical resolution, any mycobacteria that may have been dormant or hidden from the immune system could reactivate, resulting in the recurrence of clinical signs associated with mycobacteriosis. Previous studies have shown that while both radiography and CT are of benefit in the investigation of cases of feline mycobacteriosis [25,26], CT is more sensitive at detecting pulmonic changes attributed to mycobacterial infection, and that post-treatment small lesions may be missed with conventional radiography [22]. In the current study, most cats underwent radiography rather than CT, which may have not identified small regions of lung pathology at the end of treatment, resulting in treatment being withdrawn early and subsequent relapse. Data from cattle experimentally infected with *M. bovis* and treated with isoniazid showed an increase in the IFNγ response to stimulation with ESAT-6/CFP-10 after treatment was stopped, most notably in cattle with visible lesions at post-mortem examination, indicating incomplete treatment and failure to eliminate all mycobacteria [39]. One complicating factor is the dynamics of feline mycobacterial infections; although four of the five cats re-presented with clinical signs identical to their initial presentation, this could be due to reinfection from environmental sources, i.e., hunting prey, or true recrudescence of disease. Further analysis of treatment outcomes in cases of mycobacterial disease is needed. Altogether, these results suggest that attaining IGRA negativity in cats treated for mycobacterial disease is unlikely, and it does not imply any benefit regarding long-term outcomes.

There are limitations to this study, most notably the small sample size. While there were 594 individual cats that had been tested by IGRA, only 107 of these had more than one IGRA performed, and of these only 18 met the final inclusion criteria. Cats with a previous history of mycobacterial disease were excluded, as it has been shown in humans that individuals with a history of TB later presenting with non-TB disease (including bronchopneumonia and lung neoplasia) can still generate a positive IGRA result that cannot be distinguished from those with active TB [40]. Additionally, these were all naturally occurring cases that were managed in practice by the referring veterinary surgeon (RVS), with the authors overseeing case treatment remotely. Since the authors often became involved after the initial treatment choices had been made, there was no standardised approach; this was exacerbated by recent changes in treatment guidelines [2,41]. A prospective study with a defined cohort of patients, tested at set time points, would provide stronger conclusions; however, the current study provides 'real world' representations of clinical data, management of cases, and the limitations of test failures.

To conclude, serial IGRA testing to monitor the response to antimycobacterial chemotherapy is of limited benefit in the cat; however, it may be warranted to perform an end-of-treatment IGRA. Provided there is reversion to negativity, if the cat were to re-present with suspected relapse or reinfection than an IGRA could be repeated, with a positive result suggesting active mycobacterial disease. However, if the end-of-treatment IGRA were to remain positive, any further presentation with signs compatible with mycobacteriosis would require demonstration of Ziehl–Neelsen (ZN)-positive organisms. If these cannot be identified, alternative testing modalities, such as PCR or culture, would be advisable.

## 4. Materials and Methods

### *4.1. Study Population Selection*

A retrospective analysis of IGRA results from cats tested for mycobacterial disease by Biobest Laboratories, Scotland, between May 2013 and October 2019 was undertaken. Each assay had been performed as previously described [9,31]. Test submission data and results were cross-referenced with clinical data supplied by the RVS to the authors for case advice and management. This was done in line with owner consent, and the RVS was contacted for further clinical information when necessary. Individual cats with multiple IGRA results were identified. The IGRA OD values, a correlate for the amount of IFNγ secreted by T-lymphocytes in response to antigenic stimulation [30], were re-assessed in accordance with current guidelines [42], which have subsequently been adapted for feline-specific thresholds (Mitchell et al., manuscript in preparation). Briefly, for a test to pass

QC thresholds, the mean $OD_{PC}$ had to be $\geq 0.40$ and exceed the $OD_{NC}$ by at least 0.10 OD units i.e., $OD_{PC}$-$OD_{NC} \geq 0.10$; if the $OD_{PC}$ was < 0.40 it was considered a borderline pass provided $OD_{PC}$-$OD_{NC} \geq 0.20$. The $OD_{NC}$ also had to be $\leq 0.30$. The CV for duplicate control and test well OD values had to be within 30%. Individual cats without at least two tests that had passed these criteria were excluded.

Supporting clinical data was consulted for each cat (where available) to identify those with a final diagnosis of mycobacterial disease, either (1) confirmed with culture or PCR, or (2) strongly suspected (i.e., supportive histopathology and/or ZN-positive staining of tissue biopsy material, but no culture of PCR). Cats were excluded if they had insufficient clinical records or had been tested as potential in-contact cases. Cats were also excluded if they had been previously diagnosed and treated for an incidence of mycobacterial disease prior to IGRA testing. To be included in this study, all cats had to have an IGRA performed at the point of starting treatment for mycobacterial disease, and at the point of clinical resolution of active disease when treatment was stopped.

### *4.2. Qualitative Interpretation of Results*

For each cat that met the inclusion criteria described above, IGRA results were assessed to identify a positive response to any of the test antigens. For antigen positivity, the OD value for cells stimulated with PPD from *M. avium* (PPDA) and PPD from *M. bovis* (PPDB) had to exceed the $OD_{NC}$ by 0.07, whereas the OD value for the antigenic cocktail ESAT-6/CFP-10 had to exceed the $OD_{NC}$ by 0.05. A PPDA-biased response was given as $OD_{PPDA}$-$OD_{PPDB} \geq 0.05$, and a PPDB-biased response as $OD_{PPDB}$-$OD_{PPDA} \geq 0.05$ [43]. A test showing a positive response to any antigen was called positive, whereas a test lacking a positive response to all antigens was called negative. Clinical records were consulted to correlate the timing of IGRA tests with treatment. An IGRA was termed pre-treatment if it was performed within two weeks of starting treatment for mycobacterial disease, or before treatment had been instigated. The IGRA was deemed end-of-treatment if treatment had been stopped within the proceeding two weeks, or if treatment was ceased within four weeks of the test being performed. Once assigned as positive or negative, the pattern of IGRA results was interpreted as follows: those that remained positive across both tests were classified as persistently positive; those that changed from positive to negative were classed as reversions. A one-sample chi-squared test was performed to determine if the number of cats with a negative end-of-treatment IGRA differed from a hypothesised proportion of reversion of 0.33 in humans with active TB [19]. Statistical significance was set to $p < 0.05$, and data were analysed using RStudio Ver 1.2.1335 (RStudio, Inc., Boston, MA, USA) [44].

### *4.3. Quantitative Interpretation of Results*

Negative control-corrected paired $OD_{PC}$ values were compared across both testing points for each cat by plotting the average $OD_{PC}$ value for each test with a 95% confidence interval for the mean; tests with overlapping confidence intervals were considered comparable. Wilcoxon signed-rank tests were performed to compare pre- and end-of-treatment IGRA $OD_{PPD}$ and $OD_{E6C10}$ values. The selection of either PPD antigen of $OD_{PPD}$ analysis was based on demonstration of PPD-bias. Comparison of $OD_{E6C10}$ values was undertaken for cats who were IGRA-positive for this condition or were diagnosed by culture or PCR with *M. bovis*, which encodes the genes for this antigenic cocktail (unlike *M. microti*). To investigate whether cats who reverted from positive to negative at the end-of-treatment IGRA had a lower pre-treatment $OD_{PPD}$ value compared to those who remained positive, a Mann-Whitney U test was performed. Statistical significance was set to $p < 0.05$. Data were analysed using RStudio Ver 1.2.1335, and graphs were created using GraphPad Prism Ver 9.0.0 (GraphPad Software, San Diego, CA, USA).

### *4.4. Identification of Factors Associated with IGRA Reversion and Recurrence of Disease*

For cats with a valid pre- and end-of-treatment IGRA, logistic regression was performed to identify predictors of reversion to negativity at the end-of-treatment IGRA, and of disease recurrence after the cessation of antibiotic therapy. A multivariate model with all potential explanatory factors was constructed for each outcome (reversion and recurrence). A multidirectional stepwise approach was used to reduce the multivariate model to its simplest components. Statistical significance in the reduced model was set to $p < 0.05$. Logistic regression was performed using RStudio Ver 1.2.1335.

**Author Contributions:** Conceptualization, J.L.M., P.S., D.A.G.-M., and J.C.H.; methodology, J.L.M.; software, J.L.M.; validation, J.L.M.; formal analysis, J.L.M.; investigation, J.L.M.; resources, C.O., K.M., and P.B.; data curation, J.L.M.; writing—original draft preparation, J.L.M.; writing—review and editing, P.S., C.O., K.M., P.B., D.A.G.-M., and J.C.H.; visualization, J.L.M.; supervision, D.A.G.-M., and J.C.H.; project administration, J.L.M.; funding acquisition, D.A.G.-M., and J.C.H. All authors have read and agreed to the published version of the manuscript.

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**Informed Consent Statement:** Informed consent (verbal or written) was obtained from the owner or legal custodian of all animals described in this work for all procedures undertaken. No animals or people are identifiable within this publication, and therefore additional informed consent for publication was not required.

**Data Availability Statement:** The data presented here are available on request from the corresponding author. The data are not publicly available due to privacy regarding owner personal information.

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## 4.2 Discussion

From these two papers, it has been shown that by making small reductions to antigen positivity thresholds, the performance characteristics of the IGRA can be improved. Most notably, the sensitivity of the IGRA for all MTBC infections rose from 83.1% to 90.2%, and for cases of *M. bovis* infection specifically the sensitivity increased from 43.8% to 68.8%. These changes improved the agreement of the results of IGRA testing with culture and/or PCR results from weak to moderate. It was also shown that a PPDA-biased result can be observed with a range of NTM species other than just *M. avium*; these species were *M. kansasii*, *M. lepraemurium*, *M. malmoense*, and *M. smegmatis*. Regarding serial IGRA testing to monitor responses in cats treated for mycobacterial infection, the data presented in this chapter suggest this is of limited utility; 77.8% of cats remained persistently positive at the end of treatment despite clinical resolution of disease, although there was a small, but not statistically significant decrease in antigen-specific OD values between pre- and end of treatment test results. There was no association with remaining positive on the end of treatment IGRA and recurrence of disease post-treatment. Therefore, there is insufficient evidence to conclude that serial IGRA testing is of benefit in cats treated for mycobacterial infection. Additional factors to explain the recurrence of disease post-treatment were investigated, and all five cats that had recurrent disease initially presented with an abnormal pulmonic pattern on thoracic radiography. This contrasts with cats that did not have recurrent disease, where only 54% had evidence of changes associated with mycobacterial infection on pre-treatment thoracic radiographs. The presence of pulmonic lesions may therefore be a negative prognostic indicator for freedom from disease post-treatment; further studies should investigate this in more detail.

Since the IGRA became commercially available, it has been an essential part of the investigative process for working up a case of suspected mycobacterial infection in cats (O'Halloran and Gunn-Moore, 2017). In some cases, it may be more financially viable compared to culture and/or PCR (Gunn-Moore, 2014), or it may be the only test available if there are lesions that cannot be readily sampled such as choroidal granulomas (Stavinohova *et al.*, 2019) or cases presenting only with respiratory lesions (O'Halloran *et al.*, 2020). Despite its routine use, there is very limited data assessing the performance of the IGRA in cases of mycobacteriosis in cats with active disease. It was reported that approximately 80% of cats with *M. bovis* infection were positive to stimulation with the RD1 protein cocktail of ESAT-6/CFP-10 (Rhodes *et al.*, 2011), but the work presented in this study showed that only approximately 40% of cats infected with *M. bovis* gave a positive IGRA result to this antigen, substantially lower than previously thought. This means a significant number of cats with *M. bovis* infection do not respond to ESAT-6/CFP-10 and may have been misdiagnosed as being infected with *M. microti*. Reducing the OD cut-off value for ESAT-6/CFP-10 positivity increased the proportion of cats infected with *M. bovis* being positive to this antigen cocktail to nearly 70%, while maintaining excellent specificity. To provide further confidence in the adoption of these new thresholds, these values should be applied to future cases of cats that have undergone both IGRA testing for mycobacterial disease with a contemporaneous positive culture and/or PCR result.

Decisions on whether to perform repeat IGRA testing over the course of treatment or at the point of clinical resolution of disease have been based on clinical reasoning and judgement, rather than a solid evidence base. While the number of cases included in this study was small, it provides a foundation for the understanding of IGRA responses in cats that have been treated for mycobacterial infection. Comparisons to the results of serial IGRA testing in humans should be made cautiously; treatment for TB in humans is with a well-described regimen of antibiotic therapy for a defined time period (World Health Organization, 2019b), whereas the cases of feline mycobacterial disease included in this study had differing presentations of disease, were treated with various combinations of antibiotic therapy and received this for a length of time based on clinical judgement rather than a predetermined duration. Further studies should seek to describe IGRA responses in a more well-defined series of cases of feline mycobacteriosis. Despite these limitations, it would appear that there are similarities in the serial IGRA responses of cats treated for mycobacterial disease as there are with humans, where monitoring treatment responses with serial IGRA testing is no longer recommended (Clifford *et al.*, 2015). Overall, these two papers add a great deal to our knowledge regarding IGRA testing in cats with active mycobacterial disease, providing objective data from which informed decisions can be made.

However, it does not address all questions regarding the use of the IGRA in cats with potential mycobacterial infection. The IGRA is frequently used for the screening of clinically healthy cats that have been in-contact with a case of mycobacterial infection, or that have been exposed to a known risk factor (O'Halloran *et al.*, 2019, O'Halloran *et al.*, 2020). The test is used in these cases because it is the only test that can be used in cats without lesions. While the data presented in this chapter can provide us with greater confidence when interpreting IGRA results in clinically sick cats, they do not help us understand the significance of positive IGRA results in cats without overt signs of clinical disease. It is currently advised that these overtly healthy cats with a positive IGRA result should undergo further diagnostic investigations, such as thoracic radiography and abdominal ultrasonography, to try and identify any subclinical lesions (O'Halloran *et al.*, 2019). Treatment may then be instigated if changes suspicious of mycobacterial infection are identified, or it may be given prophylactically in the absence of lesions. This mirrors difficulties in TB testing in humans, as the IGRA cannot differentiate between disease states (defined in *1.1.7 Asymptomatic Cats*) (Muñoz *et al.*, 2015), and it is not fully understood why some humans with a positive IGRA result will progress to develop active disease whereas others do not (Drain *et al.*, 2018). Further work should be implemented to follow-up cases of IGRA testing in clinically healthy cats; these cases should undergo imaging to determine whether mycobacterial lesions are present, and then subsequent follow-up of whether there was progression to disease in cats that did or did not receive antimycobacterial treatment.

The work presented in this chapter focused on the statistical analysis of existing IGRA data, and while test sensitivity could be improved there remained a small number of cats that were negative on IGRA testing. Biomarkers other than IFNγ have been investigated for the diagnosis of TB in humans, and of these the most promising appears to be IP-10 (Ruhwald *et al.*, 2012). This cytokine is secreted by APCs (Dhillon *et al.*, 2007) and can be considered a downstream marker of lymphocyte activation. The use of IP-10 as a biomarker for the diagnosis of TB in animals has mostly shown positive results. The

concentration of IP-10 detected by ELISA from antigen-stimulated whole blood samples from cattle experimentally infected with *M. bovis* was approximately 10-times lower than that of IFNγ concentrations (Waters *et al.*, 2012), but other studies appear to show that detection of IP-10 may be of benefit for the diagnosis of TB in this species (Parsons *et al.*, 2016). In African buffalo measurement of IP-10 in conjunction with IFNγ increased the sensitivity for detecting infection with *M. bovis* from 80 to 100% (Bernitz *et al.*, 2019). An IP-10 release assay has also been investigated for the detection of *M. bovis* infection in warthogs and shows promise (Roos *et al.*, 2018), especially as IFNγ could not be successfully measured in this species. Given the improvement in sensitivity when measuring both IFNγ and IP-10 in African buffalo, it would be prudent to investigate whether detection of IP-10 would be of benefit for diagnosing mycobacterial infections in cats, especially for those infected with *M. bovis* that were negative to ESAT-6/CFP-10 and those that were negative to all test antigens despite a positive culture and/or PCR result.

Overall, our knowledge of the IGRA for diagnosing cases of mycobacterial infections in cats has been greatly expanded by the work presented in this chapter. Implementing the proposed new thresholds for antigen positivity could improve test sensitivity without compromising specificity, and there is evidence to suggest that IGRA responses in cats treated for mycobacterial infection reflect those seen in humans, and that attaining a negative end of treatment IGRA may both be unlikely to occur and may not confer an advantage pertaining to longer-term freedom from recurrence of disease. Further work is required to validate the IGRA as an *ante-mortem* diagnostic test for mycobacterial infections in cats, but the results shown in this chapter serve as a foundation for these studies to take place.

# Chapter 5: Recognition of rIFNγ from Felidae Species by Anti-Cat Antibodies

## Preface

The following chapter consists of one article published in Veterinary Immunology and Immunopathology. Copyright is assigned to Elsevier B.V. Author Rights are granted to use the Preprint, Accepted Manuscript and Published Journal Article for Personal Use, Internal Institutional Use and Scholarly Sharing Purposes, including submission in a non-commercially published thesis.

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## Author's Contribution

The author performed all experimental work (laboratory and computational), analysed the data, and wrote the manuscript.

## 5.1 Introduction

As shown in the previous chapter, the feline-specific IGRA is a sensitive and specific test for the diagnosis of feline mycobacterial infections, particularly for cases of TB. The availability of the IGRA has improved the ability for clinicians to identify and diagnose these infections in domestic cats, and further steps have been made to allow for IGRA testing of dogs (O'Halloran *et al.*, 2018a). This highlights that the platform of the IGRA is adaptable for use in other species but is often dependent on the availability of species-specific reagents, which can be a limiting factor for any veterinary diagnostic test.

Tuberculosis is not only a disease of domestic cats; among the Felidae family, TB has been diagnosed in many species, with reports most frequently of *M. bovis* infection in lions (Morris *et al.*, 1996, Keet *et al.*, 2000, Viljoen *et al.*, 2015). While host-species specificity is not a concern for diagnostic tests that detect the presence of the pathogen, such as mycobacterial culture (Gunn-Moore *et al.*, 2011a) or PCR (Aranaz *et al.*, 1996), immunological testing may not be possible if reagents to detect biomarkers, such as cytokines and chemokines, are not available for that species. Therefore, investigations into the cross-reactivity of antibodies from one species to detect immunological biomarkers in another, often closely related species, can be of benefit, increasing the pool of available reagents to improve our understanding of host immune responses in a wider range of veterinary species. For example, it has been shown that anti-cat antibodies can adequately detect TNF-α and IL-1β in cheetahs, however, anti-cat antibodies could not accurately measure levels of IL-6 in cheetahs (Franklin *et al.*, 2015). The requirement for the identification of more cross-reactive reagents has been highlighted, particularly for use in diagnosing infectious diseases such as TB in wildlife populations (Bernitz *et al.*, 2021).

Given the previous demonstration of cross-reactivity of anti-cat antibodies for TNF-α and IL-1β, it may be the case that anti-cat IFNγ antibodies are also suitable for the detection of IFNγ in the cheetah, as well as other felid species. A lion-specific IGRA has been investigated, using monoclonal anti-lion IFNγ antibodies raised in mice, which was able to detect IFNγ in the supernatant of mitogen-stimulated whole blood samples from lions, tigers, leopards, and domestic cats (Maas *et al.*, 2012), clearly showing promise of this assay, but also requiring the generation of reagents which may be both laborious and cost-limiting. The feline-specific IGRA has been used to screen for mycobacterial infections in lions, with a result suggestive of MTBC infection in two individuals that were also PCR-positive for MTBC DNA (Molenaar *et al.*, 2020), indicating that this feline-specific test may be of use not only in cats, but also in lions. This poses the question as to whether anti-cat IFNγ antibodies used in the IGRA can recognise IFNγ from a wider range of felid species, therefore extending the use of this test as a pan-Felidae diagnostic option.

The aim of this chapter, presented in the following paper, was to use a combination of bioinformatics and experimental techniques to investigate the characteristics of IFNγ across the Felidae family, where

data was available, and produce rIFNγ for testing with commercially available anti-cat IFNγ antibodies to determine whether the feline-specific IGRA may be beneficial for diagnosing mycobacterial infections, most notably *M. bovis*, in these species. It was also investigated whether antibodies against IFNγ from other species, namely the dog and the cow, could also detect felid rIFNγ, increasing our understanding of species cross-reactivity and the possible implications of this for future diagnostic development.

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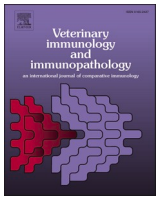

Short communication

# Recognition of recombinant interferon-gamma from Felidae species by anti-cat antibodies

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ABSTRACT

Mycobacterial infections cause a reasonable burden of morbidity and mortality in global feline populations, many of which are 'Vulnerable' or 'Endangered'. Identifying these infections may facilitate efforts to protect these animals. An interferon-gamma (IFNγ) release assay (IGRA) to diagnose mycobacteriosis in domestic cats has been adapted for use in lions; however, the development of species-specific antibodies may be laborious. Therefore, we investigated whether anti-cat IFNγ antibodies can bind to recombinant IFNγ (rIFNγ) from other Felidae species, permitting use of the feline IGRA in a wider range of felids. Unique Felidae IFNγ protein sequences and their corresponding coding nucleotide sequence were identified from online databases; plasmids with an IFNγ-gene insert were synthesised to transform *E. coli*-DH5α and subsequently transfect HEK 293 T cells to secrete rIFNγ. Enzyme-linked immunosorbent assay using a commercial anti-cat IFNγ kit was performed to detect rIFNγ from Felidae, the domestic dog and cattle. Five unique rIFNγ Felidae proteins were synthesised; anti-cat IFNγ antibodies were able to bind to all five proteins, while cross-reactivity with canine and bovine rIFNγ was negligible. This suggests that anti-cat IFNγ antibodies are sufficient for detection of IFNγ across other Felidae species, namely the lion, tiger, cheetah, cougar, Iberian lynx and the Canadian lynx.

## 1. Introduction

Mycobacteriosis is increasingly recognised in domestic cat (*Felis silvestris*) populations, but these infections are not limited to this member of the Felidae family. Mycobacterial disease has been reported in both captive and wild Felidae other than the domestic cat, where it can be a significant cause of morbidity and mortality (Keet et al., 2000).

Of the 41 Felidae species (Kitchener et al., 2017), 24 are classified as 'Near Threatened', 'Vulnerable' or 'Endangered' (IUCN, 2020). It is therefore essential as part of conservation efforts to protect these species to identify and mitigate the dangers that are posed, including the risk of disease. Mycobacterial infections have been reported in the cheetah (*Acinonyx jubatus*) (Keet et al., 2010; Kerr et al., 2020), lion (*Panthera leo*) (Morris et al., 1996; Kirberger et al., 2006; Keet et al., 2010; Miller et al., 2012, 2015; Viljoen et al., 2015; Sylvester et al., 2017; Molenaar et al., 2020), tiger (*Panthera tigris*) (Watering et al., 1972; Lantos et al., 2003; Cho et al., 2006), leopard (*Panthera pardus*) (Thorel et al., 1998; Renwick et al., 2007), cougar (*Puma concolor*) (Traversa et al., 2009), snow leopard (*Panthera unica*) (Helman et al., 1998), jaguar (*Panthera onca*) (Kapustin et al., 2006), clouded leopard (*Neofelis nebulosa*) (Cerveny et al., 2013), Iberian lynx (*Lynx pardinus*) (Briones et al., 2000; Pérez et al., 2001; Aranaz et al., 2004; Peña et al., 2006), European lynx (*Lynx lynx*) (Schmidbauer et al., 2007; Kohl et al., 2018) and the bobcat (*Lynx rufus*) (Bruning-Fann et al., 2001). Of these, the cheetah, lion, leopard, snow leopard and clouded leopard are 'Vulnerable', while the tiger and Iberian lynx are 'Endangered' (IUCN, 2020).

Diagnosing mycobacterial infections can be difficult in domestic cats, often depending on a combination of specialized mycobacterial culture (Gunn-Moore et al., 2011), molecular methods such as polymerase chain reaction (Aranaz et al., 1996), or immunological tests such as the mycobacterial antigen-specific interferon-gamma (IFNγ) release assay (IGRA) (Rhodes et al., 2008). The feline-specific IGRA is capable of identifying infections with and can discern between the two causes of tuberculosis in cats (*Mycobacterium* [*M.*] *bovis* and *M. microti*), and has some capacity to diagnose non-tuberculous mycobacterial infections (Mitchell et al., 2021). A lion-specific IGRA has been developed and may be of benefit for the diagnosis of *M. bovis* in this species, as well as for leopards and tigers (Maas et al., 2012). However, it can be laborious to

*Abbreviations:* 3D, three dimensional; cds, coding DNA sequence; DNA, deoxyribonucleic acid; ELISA, enzyme-linked immunosorbent assay; IFNγ, interferon-gamma; IGRA, interferon-gamma release assay; LB, Luria-Bertani; OD, optical density; rIFNγ, recombinant interferon-gamma.

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generate species-specific antibodies, especially given the wide range of Felidae species where mycobacterial infections have been documented. A pan-Felidae IGRA would therefore be of benefit to diagnose mycobacteriosis in animals with active disease, notably those without lesions that can be readily sampled, as well as screening of animals prior to movement as part of conservation programmes.

Cross-reactivity of antibodies with IFNγ across different species has been reported previously, with variable results (Fuller et al., 1992; Kontsek et al., 1997). Additionally, the precise epitopes to which some anti-IFNγ antibodies bind are not fully characterised, and it is thought the three-dimensional (3D) conformational shape of the protein also influences antibody binding (Novick et al., 1983; Favre et al., 1989; Zuber et al., 2016; Yasamut et al., 2019). This study aimed to identify whether anti-cat IFNγ antibodies would recognise and bind to recombinant IFNγ (rIFNγ) from different Felidae species, suggesting that the feline IGRA may be of use to diagnose mycobacteriosis in Felidae other than the domestic cat.

## 2. Materials and methods

### 2.1. Identification of IFNγ sequences and bioinformatics analysis

Complete and predicted IFNγ-coding DNA sequences (cds) and translated protein sequences from Felidae, the domestic dog (*Canis lupus familiaris*) and cattle (*Bos taurus*) (from herein also referred to as bovine) were searched for using the NCBI and UniProt databases. Nucleotide and protein sequences were aligned using Clustal Omega Multiple Sequence Alignment (Sievers et al., 2011). Pairwise alignment was calculated between sequences and pairwise identity trees constructed using Jalview Ver 2.11.1.3 to identify unique sequences (Waterhouse et al., 2009); from each unique protein sequence category a representative sequence was taken for further analysis. Signal peptide and N-glycosylation sites were predicted using SignalP-5.0 (Nielsen et al., 1997) and NetNGlyc 1.0 (Gupta and Brunak, 2002). A 3D model for each unique protein was generated using SwissModel (Waterhouse et al., 2018), aligned against the 2.0 Å bovine IFNγ model (Randal and Kossiakoff, 2000). These models were visualised and compared using PyMol.

### 2.2. Generation of IFNγ plasmids and bacterial transformation

IFNγ gene constructs were designed using the pFUSE-hIgG1-Fc1 plasmid in SeqBuilder 14 (DNASTAR Lasergene, DNASTAR, Inc, Madison, Wisconsin, USA). The insert was designed without the Fc tag in the reading frame. The IFNγ cds with an upstream ten nucleotide Kozak sequence was inserted in the multiple cloning site of the vector, between two non-overlapping restriction enzyme sites. The final nucleotide sequence insert consisting of the IFNγ cds, the Kozak sequence and the restriction enzyme cutting sites was synthesised into a pUC57-Amp plasmid (Synbio Technologies, Monmouth Junction, New Jersey, USA).

A total of 2 μg of pUC57-Amp plasmid containing the gene insert was re-suspended in 10 μL of UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, Waltham, Massachusetts, USA); 0.5 μg of reconstituted plasmid was added to 50 μL of *E. coli*-DH5α and kept on ice for 20 min, followed by heat shocking at 42 °C for 45 s before being put back on ice for two minutes. This was added to 500 μL of S.O.C. Medium (Invitrogen) and incubated at 37 °C for 60 min. Following this, 20 μL of this suspension was streaked onto LB plates supplemented with 100 μg/mL ampicillin and incubated overnight at 37 °C. A single colony was inoculated into 200 mL LB broth with 100 μg/mL ampicillin and incubated on a shaker overnight at 37 °C.

The plasmid was extracted using the ZymoPURE II Plasmid Maxiprep Kit (Zymo Research, Irvine, California, USA) following the manufacturer's guidelines. The concentration and quality of the eluted DNA was quantified using a Nanodrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and stored at −20 °C.

Digests were prepared to ligate the IFNγ gene into the pFUSE-hIgG1-Fc1 plasmid. For each digest, 1 μg of plasmid DNA (pUC57-Amp and pFUSE-hIgG1-Fc1) was added to 1 μL EcoRI (New England Biolabs, Ipswich, Massachusetts, USA), 1 μL XhoI (New England Biolabs), 2 μL 10x CutSmart Buffer (New England Biolabs), made up to 20 μL in UltraPure™ DNase/RNase-Free Distilled Water and left at 37 °C for 60 min. To each digest 3.3 μL of 6X Gel Loading Dye (Thermo Fisher Scientific) was added, and then run on a 1% agarose gel for 60 min at 130 V. Bands of the expected size for each gene insert and the digested pFUSE-hIgG1-Fc1 plasmid were cut out of the gel and the DNA extracted and purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) as per the manufacturer's guidelines. Eluted IFNγ constructs were ligated into the digested pFUSE-hIgG1-Fc1 plasmid at 1:1 and 3:1 ratios using 1 μL digested plasmid DNA, 1 μL 10X T4 DNA Ligase Reaction Buffer (New England Biolabs), 1 μL T4 DNA Ligase (New England Biolabs), 1 μL or 3 μL of IFNγ gene DNA and made up to 10 μL in UltraPure™ DNase/RNase-Free Distilled Water. This was left overnight at 4 °C and then stored at −20 °C.

Transformation of *E. coli*-DH5α with IFNγ gene-containing pFUSE-hIgG1-Fc1 plasmids was performed as described above. Transformed bacteria were streaked onto LB plates containing 1:4,000 zeocin (Invitrogen) and incubated at 37 °C overnight. Single colonies were inoculated into 10 mL LB broth with 1:4,000 zeocin and incubated at 37 °C overnight with shaking. Stocks of transformed bacteria were made by adding 500 μL of bacterial culture to 500 μL of freezing media (50 % LB broth, 50 % glycerol), snap frozen on dry ice and stored at −80 °C. Minipreps to extract the plasmid DNA using the QIAprep Spin Miniprep Kit (Qiagen) were performed as per the manufacturer's instructions, with the DNA concentration and quality quantified as above and stored at −20 °C.

Rrestriction enzyme pairs with unique cutting sites were identified for each construct using the LaserGene software to confirm the presence of the IFNγ gene in the plasmid. Digests were prepared using similar conditions as before; to 0.5 μg of plasmid DNA, 1 μL of restriction enzymes (0.5 μL per enzyme as appropriate [NotI and either NcoI or AflII (New England Biolabs)], 1 μL of 10x CutSmart Buffer and 0.1 μL of bovine serum albumin were added and made up to 10 μL in UltraPure™ DNase/RNase-Free Distilled Water. These were left for 60 min at 37 °C before addition of 1.67 μL of 6X Gel Loading Dye and then run on a 1% agarose gel for 60 min at 130 V.

For each construct, two digest-positive samples were selected for sequencing (Eurofins Genomics, Ebersberg, Germany) to confirm the presence of the correct IFNγ gene insert. Samples were prepared at 100 ng/μL in 5 μL UltraPure™ DNase/RNase-Free Distilled Water, with 5 μL of forward or reverse primers at 5 μM (forward primer pFUSE Forward UTR 5′ HTLV TGCTTGCTCAACTCTACGTC; reverse primer pFUSE Revers Fc CTCACGTCCACCACCACGCA [Merck, Gillingham, UK]). The sequenced products were aligned against the IFNγ-pFUSE-hIgG1-Fc1 constructs and for each construct one sample was selected for further processing provided there were no sequencing errors.

Frozen IFNγ-pFUSE-hIgG1-Fc1-transformed bacterial stocks were inoculated into 10 mL LB broth, then added to 200 mL LB broth containing 1:4,000 zeocin and incubated overnight at 37 °C with shaking. Maxipreps were performed as described above, with an additional step to reduce endotoxin levels; after elution of the DNA in 400 μL ZymoPURE Elution Buffer, the EndoZero Spin Column was placed in a sterile 1.5 mL Eppendorf and the elute added to the column, left for two minutes, centrifuged at 10,000 x *g* for 60 s and the concentration and quality of the eluted DNA quantified as above. Samples were stored at −20 °C.

### 2.3. Transfection of HEK 293 T cells

A volume of 1 mL of HEK 293 T cells at $1 \times 10^7$ cells/mL was added to 9 mL of filter sterilised Dulbecco's Modified Eagle Medium (Gibco, Waltham, Massachusetts, USA), supplemented with 10 % foetal bovine serum and 1% GlutaMAX™ (Gibco) and centrifuged at 400 x *g* for five

minutes. The supernatant was discarded and made back up to 1 mL in supplemented media before being added to 30 mL of supplemented media in a T175 cell culture flask. The media was gently agitated to promote cell adhesion to the flask base, followed by incubation for 24 h at 37 °C, at which point cells had reached approximately 80 % confluence.

The media was discarded, then 5 mL TrypLE™ Express (Gibco) added to dissociate cells, incubated at 37 °C for five minutes. This cell suspension was taken off and added to 5 mL supplemented media. Cells were stained with trypan blue, aliquoted at $2 \times 10^5$ cells and centrifuged at 400 x *g* for five minutes. The supernatant was discarded and the cell pellet reconstituted in 1.5 mL of supplemented media. A 24 well plate was seeded with 1.5 mL/well and the plate incubated overnight at 37 °C to achieve approximately 80 % confluence at the point of transfection.

For each construct, 2 μg of DNA was diluted in 50 μL Opti-MEM™ (Gibco) plus 1 μL Lipofectamine 2000 (Invitrogen) and incubated for five minutes at room temperature, before addition of 50 μL Opti-MEM™. From each well, 1.2 mL of media was removed and 100 μL of Opti-MEM™/Lipofectamine 2000 was added. The plate was gently agitated for 60 s and then incubated at 37 °C for four hours. A total of 1 mL of supplemented media was added to each well and the cells incubated at 37 °C for four days. For each construct transfection of HEK 293 T cells was performed in duplicate, and negative controls of Opti-MEM™/ Lipofectamine 2000 and Opti-MEM™ alone, both without DNA, were included.

Following incubation, the supernatant was harvested and pooled for each construct and control, then centrifuged at 400 x *g* for five minutes to pellet any cells present. These were stored at −20 °C prior to further analysis.

### 2.4. Enzyme-linked immunosorbent assay (ELISA)

The Feline IFNγ DuoSet ELISA (DY764, R&D, Minneapolis, Minnesota, USA) was used to screen for binding of antibodies to rIFNγ in the undiluted supernatant for all constructs and controls, in accordance with the manufacturer's guidelines. Following this, two-fold dilution series were performed for constructs to estimate the limit of detection of rIFNγ in the supernatant. Supernatants were also tested with the Canine IFNγ DuoSet ELISA (DY781B, R&D) and the Bovine IFNγ ELISA kit (MCA5638KZZ, BioRad, Hercules, California, USA), performed in accordance with manufacturer's guidelines. Samples were tested in duplicate, with a standard curve using the supplied species IFNγ run.

## 3. Results and discussion

Seventeen cds and corresponding protein sequences from eight Felidae species were identified from online databases; these species were the domestic cat (four cds), lion (four cds), cheetah (three cds), tiger (two cds), cougar (one cds), Canadian lynx (one cds), Iberian lynx (one cds) and leopard (one cds). In addition, four cds and protein sequences were identified from the domestic dog and five from cattle, respectively. Percentage identity trees showed 11 of the 17 Felidae IFNγ cds were unique; these 11 cds corresponded to five unique protein sequences (Fig. 1). These categories were termed 'Cat' (domestic cat, cheetah and cougar), 'Lynx' (Iberian lynx and Canadian lynx), 'Cheetah' (cheetah), 'Lion' (lion) and 'Tiger' (tiger, leopard and lion). Between the Felidae proteins, there was at least 97.6 % sequence identity (Table 1), compared to 86.2–86.8 % similarity with the dog IFNγ sequence and 72.5–73.7 % similarity with cattle IFNγ. All Felidae sequences were 167 amino acids in length, compared to 166 for dog and bovine IFNγ, with an additional aspartic acid residue at position 86.

For each category, a representative protein sequence and

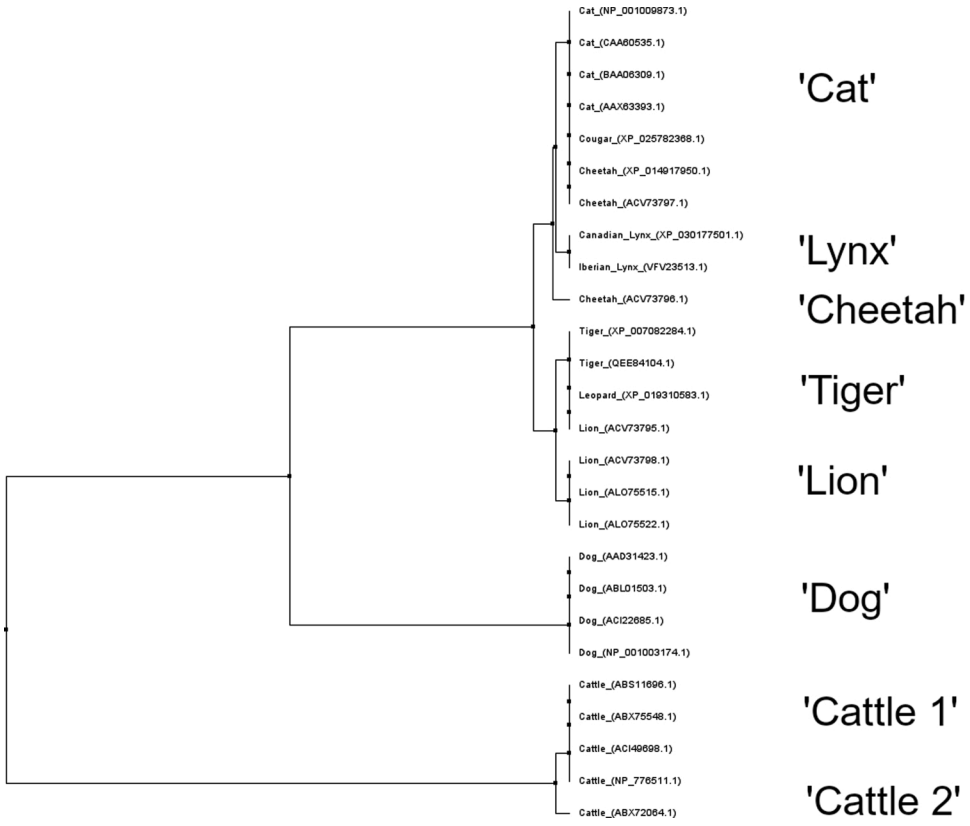


**Fig. 1.** Average distance percentage-identity tree of interferon-gamma amino acid sequences from eight different Felidae species, the dog and from cattle. Five unique interferon-gamma proteins were identified from the seventeen Felidae sequences, categorised as 'Cat' (domestic cat, cougar and cheetah), 'Lynx' (Canadian lynx and Iberian lynx), 'Cheetah' (cheetah), 'Tiger' (tiger, leopard and lion), and 'Lion' (lion).

**Table 1**
Amino acid percentage identity across the eight unique interferon-gamma proteins identified, including the five Felidae proteins. Felidae proteins differed by no less than four amino acids.

| | 'Cattle 1' | 'Cattle 2' | 'Dog' | 'Cat' | 'Lynx' | 'Cheetah' | 'Lion' |
|---|---|---|---|---|---|---|---|
| 'Cattle 2' | 99.4 | – | – | – | – | – | – |
| 'Dog' | 75.3 | 75.3 | – | – | – | – | – |
| 'Cat' | 73.7 | 73.7 | 86.8 | – | – | – | – |
| 'Lynx' | 73.7 | 73.7 | 86.2 | 99.4 | – | – | – |
| 'Cheetah' | 73.1 | 73.1 | 86.2 | 99.4 | 98.8 | – | – |
| 'Lion' | 72.5 | 72.5 | 86.2 | 98.2 | 97.6 | 97.6 | – |
| 'Tiger' | 73.1 | 73.1 | 86.8 | 98.8 | 98.2 | 98.2 | 99.4 |

corresponding cds were selected ('Cat' accession number cds NM_001009873.1, protein NP_001009873.1; 'Lynx' cds XM_030321641.1, protein VFV23513.1; 'Cheetah' cds FJ712305.1, protein ACV73796.1; 'Lion' cds KT221791.1, protein ALO75515.1; 'Tiger' cds MK463868.1, protein QEE84104.1, 'Dog' cds NM_001003174.1, protein NP_001003174.1, 'Cattle 1' cds NM_174086.1, protein NP_776511.1, 'Cattle 2' cds E276066.1, protein ABX72064.1). For the 'Cattle 1', 'Cattle 2', 'Dog', 'Lion' and 'Tiger' proteins the predicted cleavage site for the signal peptide was between residues 23 and 24, whereas for 'Cat', 'Lynx' and 'Cheetah' this was predicted to be between residues 25 and 26. An N-glycosylation site was predicted at residue 39 for all eight proteins, with an additional site at residue 107 for the five Felidae proteins (residue 106 for the 'Dog' and both 'Cattle' proteins), and within the signal peptide at residue 2 for the Felidae and 'Dog' proteins.

Models of the proteins showed minor differences in the 3D structure of the 'Cat', 'Dog' and 'Cattle 1' IFNγ (Fig. 2), with even more subtle changes between the five Felidae proteins. The five Felidae cds and the 'Dog' cds were selected for the generation of IFNγ gene-containing plasmids and subsequent transformation of *E. coli*-DH5α and transfection of HEK 293 T cells. *E. coli*-DH5α previously transformed with a bovine IFNγ gene-containing plasmid ('Cattle 1') was provided by the Immunological Toolbox (Mwangi et al., 2020).

ELISA on undiluted culture supernatant using anti-cat IFNγ antibodies showed abundant binding to all five Felidae proteins, as well as to 'Dog' rIFNγ, while binding to 'Cattle 1' rIFNγ was negligible (Fig. 3A). Two-fold serial dilutions of the supernatant from 1:12.5 to 1:32,000, was performed. The concentration of 'Cat' rIFNγ exceeded 62.5 pg/mL (the limit of detection for the kit) at a dilution of 1:32,000. There was positive detection of 'Lynx', 'Cheetah' and 'Tiger' rIFNγ at a dilution of 1:8,000 *i.e.* the optical density (OD) value at this dilution exceeded that of anti-cat IFNγ antibodies to the feline IFNγ standard at the limit of detection, while 'Lion' rIFNγ was still detected at a dilution of 1:16,000 (Fig. 3B). There was approximately 7% cross-reactivity of the canine IFNγ standard with anti-cat IFNγ antibodies compared to binding of the canine standard with anti-dog IFNγ antibodies. Anti-dog IFNγ antibodies did not cross-react with the five Felidae rIFNγ proteins, 'Cattle 1′ rIFNγ or the feline and bovine IFNγ standards, respectively. Conversely, the anti-bovine IFNγ antibodies showed 93 % cross-reactivity with the canine IFNγ standard but did not bind to the feline standard or Felidae proteins.

This study showed that the IFNγ sequence for Felidae, where data are available, is well conserved at both the coding nucleotide and amino acid level (Maas et al., 2010). Differences between 3D models of the five Felidae IFNγ proteins were subtle (data not shown); this, in turn, results in antibodies targeted against IFNγ from the domestic cat being able to recognise and bind to rIFNγ from these other members of the Felidae family. There were broadly similar levels of antibody detection of rIFNγ across the five Felidae proteins; differences in the OD values at lower dilutions could have resulted from different starting concentrations of rIFNγ in the supernatant or lower cross-reactivity of cat antibodies to these target molecules. The rIFNγ proteins were designed without an Fc-tag to minimise any conformational differences that may occur in the protein if it were present. Generation of Fc-tagged rIFNγ would provide one method for isolation and quantification of the protein, which could then be used to compare detection in ELISA against non-tagged rIFNγ as

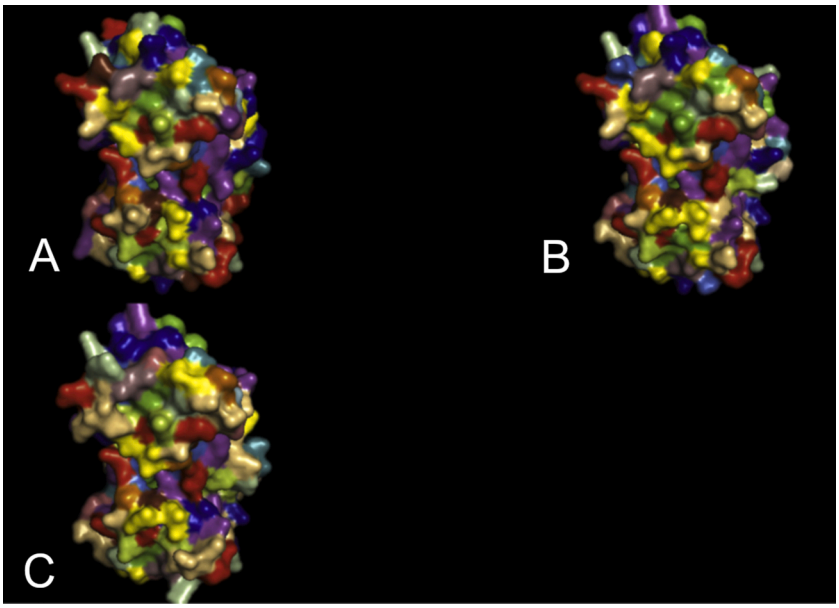


**Fig. 2.** Three-dimensional models of (A) 'Cattle 1' (B) 'Dog' and (C) 'Cat' interferon-gamma proteins predicted by SwissModel and visualised on 'show surface' setting using PyMOL to show differences in the surface structure. Each unique amino acid was assigned an individual colour to highlight differences in the sequence.

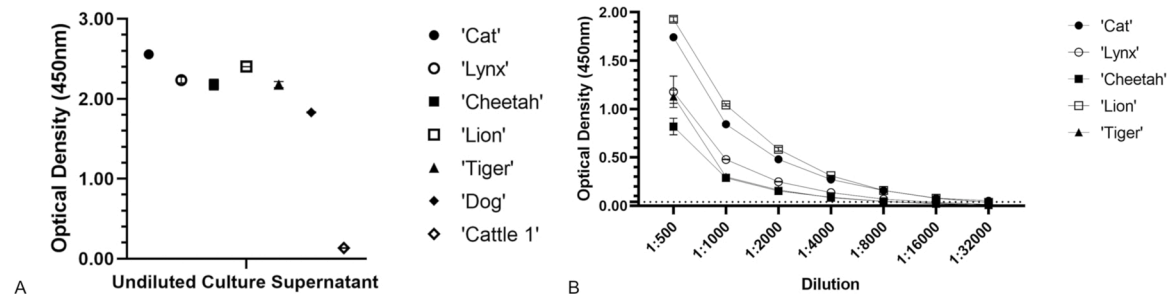


**Fig. 3.** Results of enzyme-linked immunosorbent assay (ELISA) testing using a cat interferon-gamma (IFNγ) kit for recombinant IFNγ on (A) undiluted and (B) diluted culture supernatant. All five Felidae IFNγ proteins were identified by anti-cat IFNγ antibodies with an optical density (OD) value exceeding 2.16. The mean OD value was 1.82 for 'Dog' IFNγ and 0.12 for 'Cattle 1' IFNγ. The concentration of 'Cat' IFNγ was in the range of the standard curve once diluted 1:2,000. The dotted line at OD 0.04 signifies the limit of detection of the standard curve (62.5 pg/mL). Data are plotted as average OD with error bars to show the range.

well as against cat rIFNγ to determine cross-reactivity between the proteins and cat antibodies.

Cross-reactivity was identified between the anti-cat IFNγ antibodies and 'Dog' rIFNγ, while there was no cross-reactivity of anti-dog IFNγ antibodies to the Felidae proteins or with 'Cattle 1' rIFNγ. Both the feline and canine IFNγ kits use polyclonal capture and detection antibodies; however, the lack of cross-reactivity at high concentrations of protein in the undiluted supernatant infers that the epitopes to which the anti-dog IFNγ antibodies bind are absent in the Felidae and 'Cattle' proteins. In contrast, the monoclonal anti-bovine IFNγ antibodies cross-reacted with 'Dog' rIFNγ, with an absence of binding to Felidae proteins. Felidae IFNγ appears unique amongst mammals in that it is a 167 amino acid protein (Rinderknecht et al., 1984; Ealick et al., 1991; Fuller et al., 1992; Kontsek et al., 1997; Randal and Kossiakoff, 2000; Sweeney et al., 2001); this may play a significant factor in its lack of cross-reactivity to antibodies targeted against IFNγ from other species. It also provides further evidence that cross-reactivity cannot be completely inferred from nucleotide or protein sequence data alone (Pearson, 2013).

Although limited by the number of sequences deposited in online databases, data were available from species across both subfamilies of the Felidae: the Felinae (domestic cat, cheetah, Iberian lynx, Canadian lynx, and cougar) and the Pantherinae (lion, cheetah, and leopard). Mycobacteriosis has been reported in all of these species other than the Canadian lynx. The feline IGRA has been used for screening individual lions for mycobacterial infection (Molenaar et al., 2020); however, it had not been demonstrated that the anti-cat IFNγ antibodies in this test would bind to native lion IFNγ. This work shows that, for this ELISA kit, anti-cat antibodies can detect rIFNγ from lions for which sequence data are available (Maas et al., 2010). There may be other IFNγ polymorphisms that have not yet been identified (Barker et al., 2020), for which this antibody kit may not detect IFNγ. Additionally, this kit utilises polyclonal antibodies; there are some concerns that polyclonal antibodies may lack specificity compared to monoclonal antibodies (Graham et al., 2003; Satoh et al., 2011). Determining binding responses of different monoclonal antibodies to Felidae rIFNγ may help to further characterise which epitopes these antibodies recognise and whether these antibodies could be considered pan-Felidae. Similarly, follow-up work should show that this kit can identify native IFNγ from the species tested herein. Despite these limitations, the high degree of IFNγ conservation across these eight species may mean anti-cat IFNγ antibodies can detect IFNγ from other Felidae. Therefore, the feline IGRA may be of use for diagnosing mycobacterial infections across this family, negating the requirement for the generation of species-specific reagents and testing kits.

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## Declaration of Competing Interest

The authors report no declarations of interest.

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## 5.2 Discussion

The work presented in this chapter increases knowledge of the structure and biology of IFNγ across members of the Felidae family, at both the nucleotide and protein level, as well as demonstrating the ability of a commercially available polyclonal anti-cat IFNγ antibody to bind to rIFNγ from other felid species. This could increase the options of testing for mycobacterial infections by use of the feline-specific IGRA. This chapter also showed that antibody binding cannot be predicted from sequence data alone; while the polyclonal anti-cat antibody was able to bind to the canine rIFNγ produced in this study, binding to bovine rIFNγ was negligible, while the polyclonal anti-dog antibody only recognised canine IFNγ. The monoclonal anti-bovine antibody used in this study appeared to show almost complete cross-reactivity with canine rIFNγ, with which there was 75.3% protein sequence homology between bovine and canine IFNγ, yet there was no binding to any of the five felid rIFNγ proteins, despite 72.5-73.7% shared amino acid homology.

While the role of the IGRA in domestic cats has been to diagnose mycobacterial infections in animals where disease is strongly suspected, it has also been used as a screening tool for individuals who have been in contact with a clinically sick cat, with suspected or confirmed mycobacteriosis, or for those who have been potentially exposed to mycobacteria via alternative routes such as consumption of infected food (O'Halloran *et al.*, 2019, O'Halloran *et al.*, 2020). It is therefore within reason that this test could be applied to other felid species for similar purposes, either as use as a primary or ancillary diagnostic in cases of suspected mycobacterial infection, or as a screening tool for animals in zoological collections, or prior to animal movement and introductions (Molenaar *et al.*, 2020). Other tests of CMI, namely the TST, have been used in lions to great effect, indicating that they can generate a strong cell-mediated immune response in response to infection with, or exposure to mycobacteria (Miller *et al.*, 2015, Miller *et al.*, 2019). As the IGRA is another test of CMI, it would seem likely that lions would respond adequately to this diagnostic test, and a clear need to increase the repertoire of available diagnostics for mycobacterial infections in these species, as well as other felids has been identified (Maas *et al.*, 2012, Kerr *et al.*, 2020, Bernitz *et al.*, 2021). While further work would be required to demonstrate that the feline IGRA could be used to diagnose mycobacterial infections in other felid species, these data show that in principle this test should be suitable as anti-feline antibodies can detect rIFNγ from a range of other Felidae. Testing samples from species such as the lion with confirmed mycobacterial infection to determine whether these antibodies can detect native IFNγ would be the next step toward validating this assay for use in other species. It has already been demonstrated that lion PBMCs respond best to the same mitogen as used in the feline IGRA (Rhodes *et al.*, 2008a, Maas *et al.*, 2012), and testing of two lions with PCR-confirmed MTBC infection using the feline IGRA gave positive results suggestive of *M. bovis* or MTBC infection (Molenaar *et al.*, 2020), albeit using a different anti-cat antibody than the one tested herein (Mitchell *et al.*, 2021e). Therefore, further work to characterise the ability of other antibodies, in particular monoclonal antibodies, should be performed to elucidate the cross-reactivity profile of these other antibodies, which may recognise different epitopes which are not conserved across Felidae IFNγ.

While this study investigated the ability of anti-cat antibodies to bind to rIFNγ from other felid species, the degree of cross-reactivity could not be truly quantified. The IFNγ gene insert was designed without the crystallisable fragment (Fc) tag in the reading frame in case this altered the 3D conformation of the protein or could act in other ways that would interfere with antibody binding. Therefore, the protein in each supernatant could not be purified and subsequently quantified. It was possible to calculate the degree of cross-reactivity of antibodies with rIFNγ in the harvested supernatant for the 'Cat', 'Dog' and 'Cattle 1' constructs, as a protein standard for each of these was available. Calculations of cross-reactivity for the other felid species would rely on the assumption that all proteins were secreted into the supernatant in equal amounts. Determining cross-reactivity would also be dependent on the availability of protein standards and species-specific anti-IFNγ antibodies, which are not readily available, hence the requirement to identify reagents which work across species. Had Fc-tagged proteins been synthesised, this would provide a means for extraction of the protein from the supernatant so that the concentration of each could be determined, and then a dilution series performed for each protein and the degree of cross-reactivity calculated compared to the binding of anti-cat antibodies to cat rIFNγ. This could be important to examine in future studies.

To conclude, this work provides the largest comparison and characterisation of IFNγ from felid species to date and shows that anti-cat IFNγ antibodies are capable of binding to rIFNγ from other members of the Felidae family. It also shows that antibodies against IFNγ from the dog and the cow do not bind to felid rIFNγ, and that cross-reactivity of species-specific antibodies cannot be predicted on sequence data alone. Therefore, when planning to use immunological reagents in a species other than that which the reagent, in this case an antibody, is raised against, their use should be comprehensively validated to confirm cross-reactivity. Overall, it would appear that the feline IGRA may indeed be suitable as a pan-felid IGRA, expanding the options of diagnostic tests available for mycobacterial disease, and in particular *M. bovis* infection, in these threatened species. This could help conservation efforts with testing of animals prior to movement between zoological collections (Molenaar *et al.*, 2020), or the re-introduction of species into former native habitats (Ovenden *et al.*, 2019), as well as testing of wildlife populations to monitor the threat and spread of disease in vulnerable populations (Peña *et al.*, 2006, Sylvester *et al.*, 2017).

# Chapter 6: Development of an ELISA for the Serodiagnosis of Feline TB

## 6.1 Introduction

Tests of CMI, such as the IGRA, remain the mainstay for the immunological diagnosis of TB, and as demonstrated in the two previous chapters can play an integral part in diagnosing and differentiating cases of TB in cats, as well as having some scope for identifying NTM infections. However, CMI only represents one arm of the host's response to mycobacterial infections.

Recently, there has been renewed interest in the role of the humoral immune response in cases of mycobacterial infection, and this topic has been extensively reviewed (Achkar *et al.*, 2015, Rijnink *et al.*, 2021). Studies with CD20+ B-cell depleted cynomolgus macaques (*Macaca fascicularis*) experimentally infected with *M. tuberculosis* appear to suggest a role for B-cells in modulating cytokine production, the degree of inflammation, and in influencing the bacterial burden of lesions (Phuah *et al.*, 2016). Additionally, in mice vaccinated with a TB antigen B-cells were reported to play a role in antigen presentation and to help to induce memory 'precursor' effector cells (Dubois Cauwelaert *et al.*, 2016). Immunohistochemical exploration of mycobacterial granulomas have demonstrated the presence of B-cells, including in cats (Chapters 2 and 3 of this thesis) (Kipar *et al.*, 2003, Mitchell *et al.*, 2021b), where they may form ectopic follicles. The exact role of these follicles is unclear, but they may help to modulate the local inflammatory response and control numbers of intralesional bacteria (Maglione *et al.*, 2007). Antibodies may also play a role in reducing bacterial numbers. Although mycobacteria are intracellular pathogens, they undergo periods where they are extracellular (Abebe and Bjune, 2009); therefore, at these points they may be susceptible to antibody-mediated mechanisms of defence. Opsonisation of *M. tuberculosis* has been shown to overcome inhibition of phagolysosomal fusion, restricting mycobacterial growth and increasing intracellular killing of this pathogen (Chen *et al.*, 2020), while PPD-specific IgG has been shown to increase Natural Killer cell (NK)-mediated cellular cytotoxicity, particularly in individuals with LTBI (Lu *et al.*, 2016). It has been suggested that glycosylation of antibodies can modulate their activity and may provide insights into different disease states (Lu *et al.*, 2016). However, the focus of antibodies in cases of mycobacterial infection has largely been in their use as diagnostic biomarkers, in human and animal populations (Lyashchenko *et al.*, 1998b, Gounder *et al.*, 2002, Miller *et al.*, 2012, Wang *et al.*, 2018).

Antibody-based diagnostics are attractive in the terms of their simplicity and ease of flexibility, they can be used as point-of-care tests and for some formats a result can be obtained in 15 minutes or less (Greenwald *et al.*, 2003). In humans, antibody tests are available for both TB and leprosy (Anderson *et al.*, 2008, Spencer and Brennan, 2011), and serodiagnostic tests have also been investigated for *M. avium* infections (Kitada *et al.*, 2002). Despite the availability of such tests, the World Health Organization have concluded that such tests should not be used for the diagnosis of pulmonary or extra-pulmonary TB in humans, given inconsistent and unreliable test results, with varying sensitivity and specificity, and unacceptable rates of false-positive and false-negative results (Steingart *et al.*, 2011). However, antibody-based diagnostics are still often used in cases of suspected TB, particularly in

endemic areas where access to other tests may be limited and serological testing offers an attractive and affordable diagnostic option (Steingart *et al.*, 2012).

In animals, antibody-based diagnostics have been developed, particularly for the diagnosis of TB in cattle (Waters *et al.*, 2011a, Fontana *et al.*, 2018), but these have been adapted and extended for use in other species such as the Eurasian badger, white-tailed deer (*Odocoileus virginianus*), brushtail possums (*Trichosurus vulpecula*) and wild boar (Greenwald *et al.*, 2003, Lyashchenko *et al.*, 2008). In addition, antibody-based tests are used for the diagnosis of other mycobacterial diseases of importance such as infection with MAP, that results in Johne's Disease (Nielsen *et al.*, 2001, Lilenbaum *et al.*, 2009). As in humans, these antibody based diagnostic tests are typically ELISAs or immunochromatographic rapid tests, although a chemiluminescent assay for the diagnosis of *M. bovis* in cattle has been described (Whelan *et al.*, 2008, Whelan *et al.*, 2010, Whelan *et al.*, 2011). Alternative approaches, such as the MAPIA (Lyashchenko *et al.*, 2000), fluorescence polarisation assays (Surujballi *et al.*, 2002), and Luminex-based assays (Khan *et al.*, 2008, Fontana *et al.*, 2018) remain largely limited to research settings. Concerns with test sensitivity and specificity have also been raised (Amadori *et al.*, 1998, de La Rua-Domenech *et al.*, 2006), with a recent meta-analysis of antibody-based diagnostic tests for bTB suggesting a range of sensitivity from 44-91% and specificity of 88-100%, depending on the methodology applied (Nuñez-Garcia *et al.*, 2018). There are also concerns when testing for cases of TB pertaining to antibody cross-reactivity with antigens of other mycobacterial species, particularly if crude antigens such as culture filtrates, whole cell sonicates and tuberculin are used (Plackett *et al.,* 1989, Lyashchenko *et al.,* 2008, Whelan *et al.,* 2008), hence the increasing use of more specific antibody targets, such as the use of single antigen or polyproteins (Green *et al.,* 2009, Lyashchenko *et al.,* 2021b). Despite this, there are some potential benefits of antibody-based diagnostic tests in veterinary species. For example, cattle may become anergic and no longer responsive to tests of CMI such as the TST and/or the IGRA, so the use of ancillary antibody-based diagnostic tests may help to identify *M. bovis*-culture positive animals so that they can be removed from the herd (Plackett *et al.*, 1989, Waters *et al.*, 2011a, Waters *et al.*, 2017, Fontana *et al.*, 2018, Watt *et al.*, 2019, Watt *et al.*, 2020). Another advantage of antibody-based tests, in particular rapid tests, is their use in the field, such as for the identification of infection with *M. bovis* in wildlife species such as the badger (Greenwald *et al.*, 2003). This allows for singular testing of an animal with a read-out available in minutes, without the need to re-identify and re-sample the animal.

Unlike the IGRA, no commercially available feline-specific antibody-based diagnostic tests exist. It is therefore unsurprising that there is a paucity of information on the feline humoral immune response to mycobacterial infections, and the utility of antibody testing in this species. The largest study of antibody testing in cats showed promise for the use of rapid tests based on MPB83 and ESAT-6/CFP-10 (TB Stat-Pak and DPP VetTB [Chembio Diagnostics, Medford, New York, USA]), with 90% of cats (n = 9/10) with *M. bovis* infection testing positive in either diagnostic kit (Rhodes *et al.,* 2011). However, each assay was less sensitive for *M. microti* infections (TB Stat-Pak, n = 6/13, 46.2%, DPP VetTB, n = 5/12, 41.2%). The results of MAPIA testing were less promising, with positive results to MPB83 and MPB70 observed in cats with NTM infections and in healthy controls. In addition, positivity to MPB64 (NTM-

infected cats) and MPB59 (healthy controls) was also noted (Rhodes *et al.*, 2011). A study of five cats in one household, where one cat was clinically sick with signs compatible with mycobacterial infection, showed two seropositive individuals to all three tests used (TB Stat-Pak, DPP VetTB and MAPIA). This included the clinically sick cat, as well as one individual that was later shown on thoracic radiography to have radiographic signs suggestive of mycobacterial disease (Ramdas *et al.*, 2015). Follow-up testing showed a decrease in antibody levels in these two cats while on treatment, while the remaining three remained seronegative; a decrease in antibody levels has been identified in humans treated for MAC-pulmonary disease and has been suggested to correlate with successful treatment (Kitada *et al.*, 2005, Kitada *et al.*, 2008).

A third study investigated the use of an in-house ELISA using PPDA and PPDB. This gave a positive result to PPDB in 20% of cats (n = 4/20); these four positive animals were the offspring of a cat with confirmed *M. bovis*-infection. However, no TB-associated lesions were identified in any of these ELISA positive individuals, and a similar response was reported to PPDA (Kaneene *et al.*, 2002). Finally, one study using cats injected with a heat killed preparation of *M. bovis* (termed '*M. bovis*-sensitinogen') were tested with both a rapid test and by MAPIA (Fenton *et al.*, 2010). Four out of six cats were positive on the rapid test (to antigens ESAT-6, CFP-10, Acr1 and MPB83); however, this was seen following intradermal injection with PPDB to determine TST responses, which has been shown to boost antibody responses in cattle (Hanna *et al.*, 1992, Casal *et al.*, 2014, Waters *et al.*, 2015). A positive antibody response on MAPIA testing was reported for the crude *M. bovis* culture filtrate (MBCF) antigen, but 10.2% of control cats (n = 5/49) were also positive to MBCF; whereas no antibody responses to PPDB were identified on MAPIA. There were less consistent positive results to the 16kDa protein, MPB83, and the 16kDa/MPB83 fusion protein. Where positive antibody responses were recorded, these were boosted following intradermal injection with PPDB for TST (Fenton *et al.,* 2010). Overall, these studies suggest there may be some scope in antibody testing for the diagnosis of mycobacterial infections in cats, in particular TB, but further work is required to achieve a reliable test, to identify antigens that elicit a strong humoral response in cats, and to develop a test which can differentiate between the two causes of TB in this species, *M. bovis* and *M. microti*.

The aim of this chapter was to investigate whether antibody testing could play a role in the diagnosis of mycobacterial infections in naturally infected cats using a newly developed and optimised ELISA. This allowed for initial investigation of the ELISA as a testing platform for a small number of confirmed mycobacteria infected and non-infected control feline samples, to maximise signal and minimise background noise and confirm the final testing protocol. Secondly, to investigate any effect of matrix type on the ELISA paired blood samples from cats were tested using the finalised protocol. This would determine whether there were any potential discrepancies between testing different sample matrices (*e.g.,* serum *vs.* plasma), and whether the assay should only be performed on a particular set of sample types. Test cut-offs for antigen positivity were established using ROC curve analysis comparing ELISA OD values between cats with confirmed mycobacterial infection and non-infected controls. These values then allowed for the determination of the test sensitivity and specificity. This allowed for evaluation of the ELISA, comparing it with known performance characteristics of other diagnostic tests

for feline mycobacterial infections, namely specialist mycobacterial culture and IGRA (Chapter 4). Subsequently, this allowed for the testing and classification of samples from cats as seropositive or seronegative where mycobacterial infection was suspected due to clinical disease, where there was known contact with an infected animal, or if there was another route of potential exposure. Results of antibody testing were compared against those of CMI *i.e.*, IGRA, where available, to determine whether this ELISA could play an ancillary role in the diagnosis of mycobacterial infection in some cats.

## 6.2 Materials and Methods

### 6.2.1 Sample Acquisition and Storage

Ethical approval for this study was granted by The University of Edinburgh Veterinary Ethical Review Committee (approval no. 79 14).

Opportunistically obtained archived remnant samples (separated serum, heparin plasma, or ethylene diamine tetra-acetic acid [EDTA] plasma) submitted to the Royal (Dick) School of Veterinary Studies and The Roslin Institute, University of Edinburgh, or Biobest Laboratories Ltd. were used in this study. Samples were obtained from cats undergoing diagnostic investigation by their PVS for suspected mycobacterial disease following discussions with the Companion Animal Mycobacteria Advice Service at The Roslin Institute. These samples were stored at -80°C prior to testing. Clinical data provided by the PVS were collated to provide further information for each sample and case. Samples submitted following the implementation of a full antimycobacterial therapy protocol *e.g.*, rifampicin, a macrolide/azalide, and a fluoroquinolone, were not included in the final study following protocol optimisation.

Remnant heparin plasma samples from specific-pathogen-free (SPF) cats donated from the Waltham Centre for Pet Nutrition were used as negative control samples (n = 20). These samples had been donated for use in a previous study (O'Halloran *et al.*, 2018b). All cats had undergone a routine annual health check including physical examination, and haematological and serum biochemical testing, and were required to be found to be healthy, with no abnormalities detected on bloodwork. Aliquoted samples were sent to The Roslin Institute and stored at -80°C prior to testing.

Remnant samples from three cats belonging to members of the Companion Animal Mycobacteria Advice Service, undergoing routine health checks were obtained and stored at -80°C prior to testing. There was no clinical suspicion for mycobacterial infection in these three cats. Normal cat serum which had been stored at -20°C, kindly provided by Dr Conor O'Halloran (The Roslin Institute) was also made available for testing.

## 6.2.2 ELISA Development and Optimisation

A comparative PPD ELISA (PPDB-PPDA, ΔPPD) protocol for the detection of antibodies in deer and pig sera specific to PPDB, developed by Dr Shelley Rhodes (APHA), was used as the basis for a feline mycobacteria antibody ELISA. Briefly, a Nunc MaxiSorp™ 96-well plate (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was coated with 50µL/well of antigen diluted in coating buffer, and the plate covered and incubated overnight at 4°C. The plate was washed six times with phosphate-buffered saline (PBS) and 0.05% Tween® 20 (P1370, Sigma-Aldrich, St Louis, Missouri, USA) using a SkanWasher 400 (Skantron, Molecular Devices, Sunnyvale, California, USA); all subsequent wash steps were performed in this manner. Following this, wells were blocked with 200µL of 20% unsweetened soya milk (Alpro, Wevelgem, Belgium) diluted in PBS, covered, and left for two hours at RT. After washing, samples diluted 1:50 in 20% soya/PBS were added at 50µL/well, the plate covered, and left for two hours at RT. The plate was washed, and 50µL/well of Protein A conjugated to horseradish peroxidase (HRP) diluted in 5% soya/PBS was added, the plate covered, and left for one hour at RT. After washing, 100µL/well of 1-Step™ Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific) was added and the plate left for five minutes at RT on a shaker, followed by the addition of 100µL/well of 0.5M sulfuric acid. The OD value for each well was read at 450nm and 630nm using a Cytation 3 Imaging Reader (BioTek, Winooski, Vermont, USA), results captured on Gen5 Microplate Reader and Imaging Software (BioTek) and exported to an Excel (Microsoft, Redmond, Washington, USA) spreadsheet for further analysis.

All samples were tested in duplicate for each antigen, and to pass control thresholds the coefficient of variation (CV) between wells had to be ≤ 20%. If the CV exceeded 20%, a repeat ELISA was performed on that sample.

### 6.2.2.1 Antigen Dilution Optimisation

An antigen dilution series was performed to determine the final dilutions to be used in the ELISA. Antigens were diluted in 0.05M carbonate-bicarbonate (C-BC) buffer, pH 9.6 (C3041, Sigma-Aldrich); PPDA 2500 international units (IU) and PPDB 3000 IU (Prionics Lelystad B.V., Lelystad, The Netherlands), both kindly donated by Dr Shelley Rhodes, were diluted 1:100, 1:500, 1:1,000 and 1:2,000, while ESAT-6/CFP-10 (Lionex Diagnostics and Therapeutics GmbH, Braunschweig, Germany), provided by Professor Jayne Hope (The Roslin Institute), was diluted 1:50 (20µg/mL), 1:100 (10µg/mL), 1:200 (5µg/mL), and 1:500 (2µg/mL). Antibody detection was achieved using Protein A-HRP conjugate (P8651, Sigma-Aldrich) diluted 1:20,000 in 5% soy/PBS. Serum or plasma samples from three cats with culture-confirmed *M. bovis* infection and three uninfected cats were tested to determine optimum antigen dilutions.

#### 6.2.2.2 Coating Buffer and Protein A-HRP Optimisation

Having selected an appropriate dilution for all three test antigens, different coating buffers and dilutions of Protein A-HRP were compared. Antigens were diluted in 0.05M C-BC buffer or PBS, respectively. For both coating buffers, detection of bound matrix antibodies was achieved using dilutions of Protein A-HRP at 1:20,000, 1:40,000 and 1:80,000. Serum or plasma samples from five cats with culture- or PCR-confirmed mycobacterial infection (*M. bovis* = 3, *M. avium* = 1, *M. microti* = 1), including the same three *M. bovis*-culture positive cats and three uninfected cats as in 6.2.2.1 were tested for coating buffer and Protein A-HRP optimisation.

### 6.2.3 Correlation of OD Values across Matrices

Following optimisation of the protocol, ELISAs were performed on paired samples to investigate the potential effect of sample matrix (serum, heparin plasma and EDTA plasma) on OD value. Four cats had serum, heparin plasma and EDTA plasma samples available for testing, a further four cats had both serum and EDTA plasma samples, one cat had serum and heparin plasma samples, and one cat had heparin plasma and EDTA plasma samples available for testing. Comparisons were therefore made on the following: serum *vs.* EDTA plasma (n = 8), serum *vs.* heparin plasma (n = 5) and EDTA plasma *vs.* heparin plasma (n = 5) for ΔPPD and ESAT-6/CFP-10.

### 6.2.4 Determination of ELISA Sensitivity and Specificity

To calculate test sensitivity and specificity, and to determine a positive cut-off value for testing further samples, ELISAs were performed on samples from cats with culture- or PCR-confirmed mycobacterial infection, and uninfected *i.e.,* 'negative' cats. Samples were available from 32 cats with mycobacterial infection (*M. bovis* = 12, *M. microti* = 5, unclassified MTBC = 10, *M. avium* = 2, *M. kansasii* = 1, *M. lepraemurium* = 1, *M. smegmatis* = 1) and 24 uninfected cats (SPF cats = 20, owned, uninfected pet cats = 3, normal cat serum from donor = 1).

### 6.2.5 Testing of Additional Feline Samples

Samples from cats taken on suspicion of mycobacterial infection, but without culture- or PCR-confirmation, were divided into five groups. Group 1 (n = 28) consisted of samples from cats with ZN-positive histopathology or cytology, morphologically consistent with mycobacteria. Group 2 (n = 42) consisted of samples from cats where mycobacterial infection was strongly suspected based on a

combination of clinical signs, supportive histopathology or cytology (granulomatous-to-pyogranulomatous inflammation), but without positive ZN-staining, and/or a positive response to specific antimycobacterial therapy. Group 3 (n = 7) comprised samples from cats where mycobacterial infection was less likely on reflection of supporting clinical data, or an alternative diagnosis was reached. Group 4 (n = 68) was samples from cats with insufficient supporting clinical data available to determine their likely infection status *i.e.,* unknowns. Cats that were sampled in the absence of overt clinical disease but had been in contact with a case of feline mycobacteriosis or had consumed a raw food diet associated with an outbreak of *M. bovis*-infection (O'Halloran *et al.*, 2019, O'Halloran *et al.*, 2020) were assigned to Group 5 (n = 41). Within each group, cats were subcategorised by the corresponding IGRA result interpreted in accordance with updated guidelines where raw data were available (Chapter 4) (Mitchell *et al.*, 2021e); otherwise, results were interpreted as per the test report.

### 6.2.6 Statistical Analyses

Spearman's rank correlation coefficient was calculated for paired OD values between different sample matrices (serum *vs.* EDTA plasma, serum *vs.* heparin plasma and EDTA plasma *vs.* heparin plasma). To determine the degree of bias between OD values from paired samples of different matrices, Bland-Altman analysis was performed.

Determination of antigen positive cut-off values for the ΔPPD and ESAT-6/CFP-10 ELISAs was performed by ROC curve analysis; the ΔPPD cut-off value was calculated using the OD values from cats with culture- or PCR-confirmed MTBC infection ('positives') against uninfected ('negative') cats, while the ESAT-6/CFP-10 cut-off value was determined from the OD values of cats with culture- or PCR-confirmed infection with RD1 positive mycobacteria ('positive') against uninfected ('negative') cats. Cut-off values were selected and applied back to the set of results from confirmed mycobacterial cases to determine whether these values were appropriate when taking the results of NTM-infected cats into account. Established cut-off values were then applied to the OD values from the five groups of samples taken from cats without culture- or PCR-confirmation of mycobacterial infection. All statistical analyses were performed using GraphPad 9.2.0 (GraphPad Software, San Diego, California, USA).

## 6.3 Results

### 6.3.1 ELISA Optimisation

For the ΔPPD ELISA, across all four dilutions of the antigens there was an observable difference in OD values between two cats infected with *M. bovis* compared to the three uninfected cats. In addition, one

*M. bovis* infected cat had low OD values similar to the uninfected cats (Figure 6.1 A). The ΔPPD OD values in the two reactive *M. bovis*-positive cats decreased as each antigen became more dilute, but the step-fold decrease in OD values was not proportional to the change in dilution *i.e.*, the OD value did not half when the antigen dilution doubled. The OD values for the three uninfected cats and one *M. bovis*-positive cat were consistently low across all four antigen dilutions. Dilutions of PPD antigens of 1:2,000 were selected given the maintenance of good differentiation between positive and uninfected cats.

In contrast, while all three *M. bovis*-positive cats showed OD values greater than the three uninfected cats for the ESAT-6/CFP-10 ELISA, the OD values for two of the *M. bovis*-infected individuals were very low, while one showed substantially higher OD values than the uninfected control cats (Figure 6.1 B) The OD values for all six cats were consistent across all four concentrations of this antigen. A coating concentration of 5µg/mL was selected for application to a larger subset of samples to determine the utility of this antigen in an ELISA format for diagnosing cases of feline *M. bovis* infection.

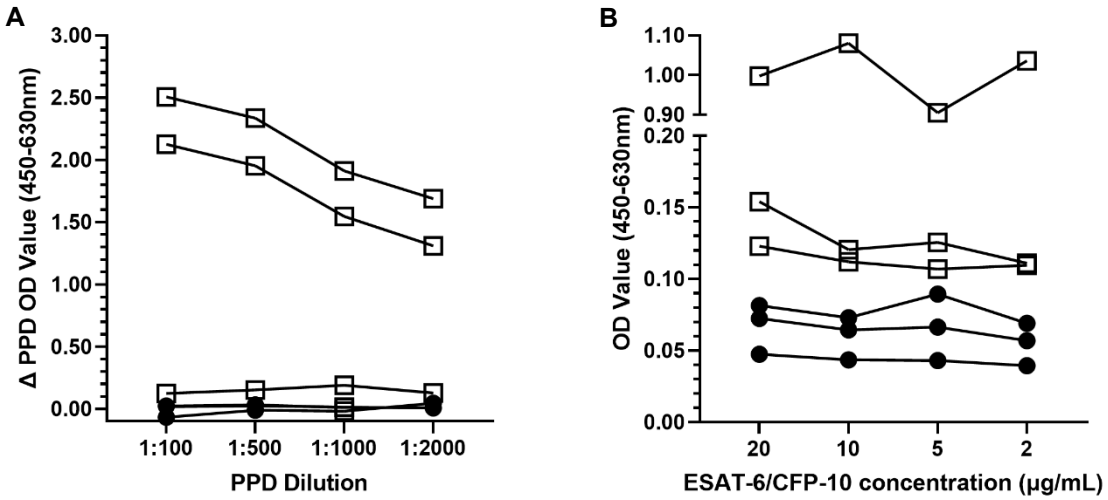


**Figure 6.1:** Antigen dilution series for ΔPPD (A) and ESAT-6/CFP-10 (B) for the development of an ELISA to diagnose cases of feline TB. Dilutions of PPD antigens at 1:2,000 and a concentration of ESAT-6/CFP-10 at 5µg/mL were used for subsequent test optimisation.

Open squares = *M. bovis*-infected cats. Closed circles = uninfected cats.

Having selected the final coating antigen dilutions (PPDA and PPDB, 1:2,000 each) or concentration (ESAT-6/CFP-10, 5µg/mL), the effect of the antigen coating buffer and the dilution of Protein A-HRP were investigated on a larger cohort of samples including those from cats with *M. microti* and *M. avium* infections. For the ΔPPD ELISA, across all dilutions of Protein A-HRP the OD values were consistent regardless of the antigen coating buffer used, although the OD values were slightly higher for two *M. bovis*-infected cats (open squares) when PPDA and PPDB were diluted in PBS compared to C-BC buffer (Figure 6.2 A). A notable difference in OD values when PBS was used compared to C-BC buffer

was not observed for the one *M. microti*-infected cat (closed squares). There was a greater effect of Protein A-HRP dilution on OD values; while differentiation was maintained between two of the cats infected with *M. bovis* and the one *M. microti*-positive cat to the remaining five cats (three uninfected [closed circles], one positive for *M. avium* [open triangles] and one infected with *M. bovis* [open squares]), it was less distinct at a dilution of 1:80,000, and the overall OD values for the three reactive cats were quite low (Figure 6.2 A).

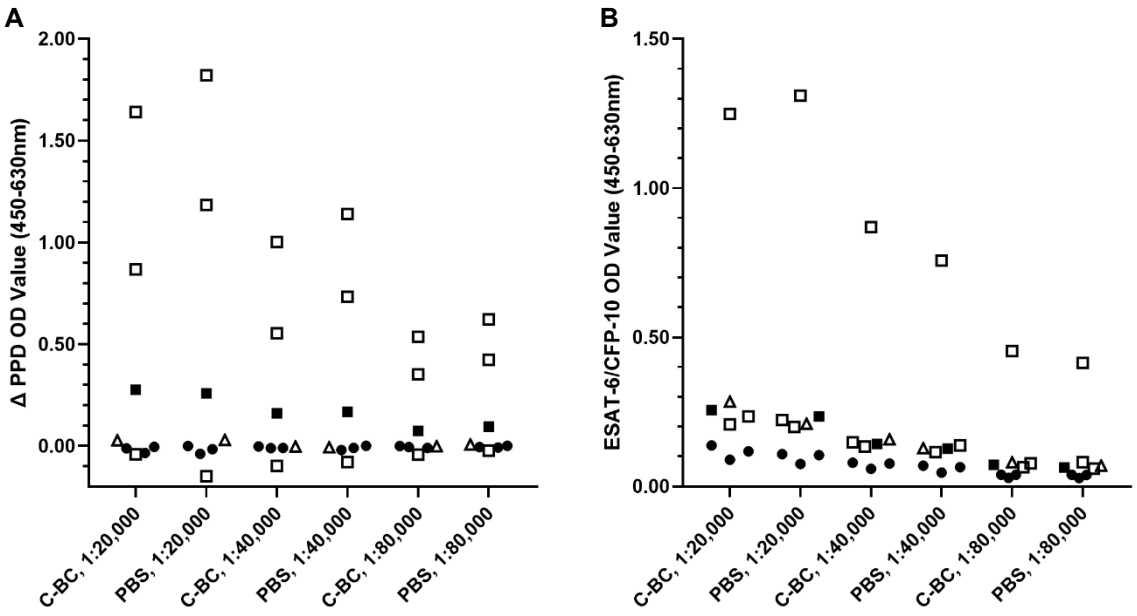


**Figure 6.2:** Comparison of antigen coating buffer and Protein A-HRP dilution for ΔPPD (A) and ESAT-6/CFP-10 (B) ELISA. Optical density values were broadly consistent regardless of the choice of coating buffer, whereas values were greatly decreased at greater dilutions of Protein A-HRP.

Open squares = *M. bovis*-infected cats. Black squares = *M. microti*-infected cats. Open triangles = *M. avium*-infected cats. Closed circles = uninfected cats.

For the ESAT-6/CFP-10 ELISA, a similar pattern was noted regarding both choice of coating buffer and dilution of Protein A-HRP (Figure 6.2 B), although the OD values observed when PBS was used as the antigen coating buffer were not always higher than those obtained when the C-BC buffer was used. Given the lack of apparent difference between antigen coating buffers, and the loss of differentiation between reactive and non-reactive samples with greater dilutions of Protein A-HRP, C-BC buffer was selected for diluting antigens and coating the ELISA plate, and the optimum dilution of Protein A-HRP was determined to be a dilution of 1:20,000.

## 6.3.2 Comparison of OD Values across Biological Matrices

Having optimised the protocol, the ELISA was run on 24 samples from 10 cats to determine how well the OD values correlate across biological matrices, as well as to calculate the amount of bias present. Correlation results are shown in Figure 6.3 and Table 6.1.

Broadly, OD values correlated well across all sample matrices for both the ΔPPD (Figure 6.3 A-C) and ESAT-6/CFP-10 ELISAs (Figure 6.3 D-F), with values of Spearman's rho ≥ 0.70 (Schober *et al.*, 2018). While perfect correlation was recorded between some matrices (EDTA plasma and heparin plasma for both the ΔPPD and ESAT-6/CFP-10 ELISAs), there were discrepancies in OD values. On average, the OD values for both ΔPPD and ESAT-6/CFP-10 were greater for EDTA plasma samples compared to serum and heparin plasma, while the OD values for serum were greater than those for heparin plasma for both ELISAs (Table 6.1).

These results suggest that OD values are broadly consistent regardless of the matrix used, although small differences may be present which could affect the classification of a test result as positive or negative if it is close to a pre-determined threshold. Given these results, it was decided that all samples would be suitable for further analysis, with a preference for serum or heparin plasma where these were available given these matrices had the smallest amount of bias for both ELISAs.

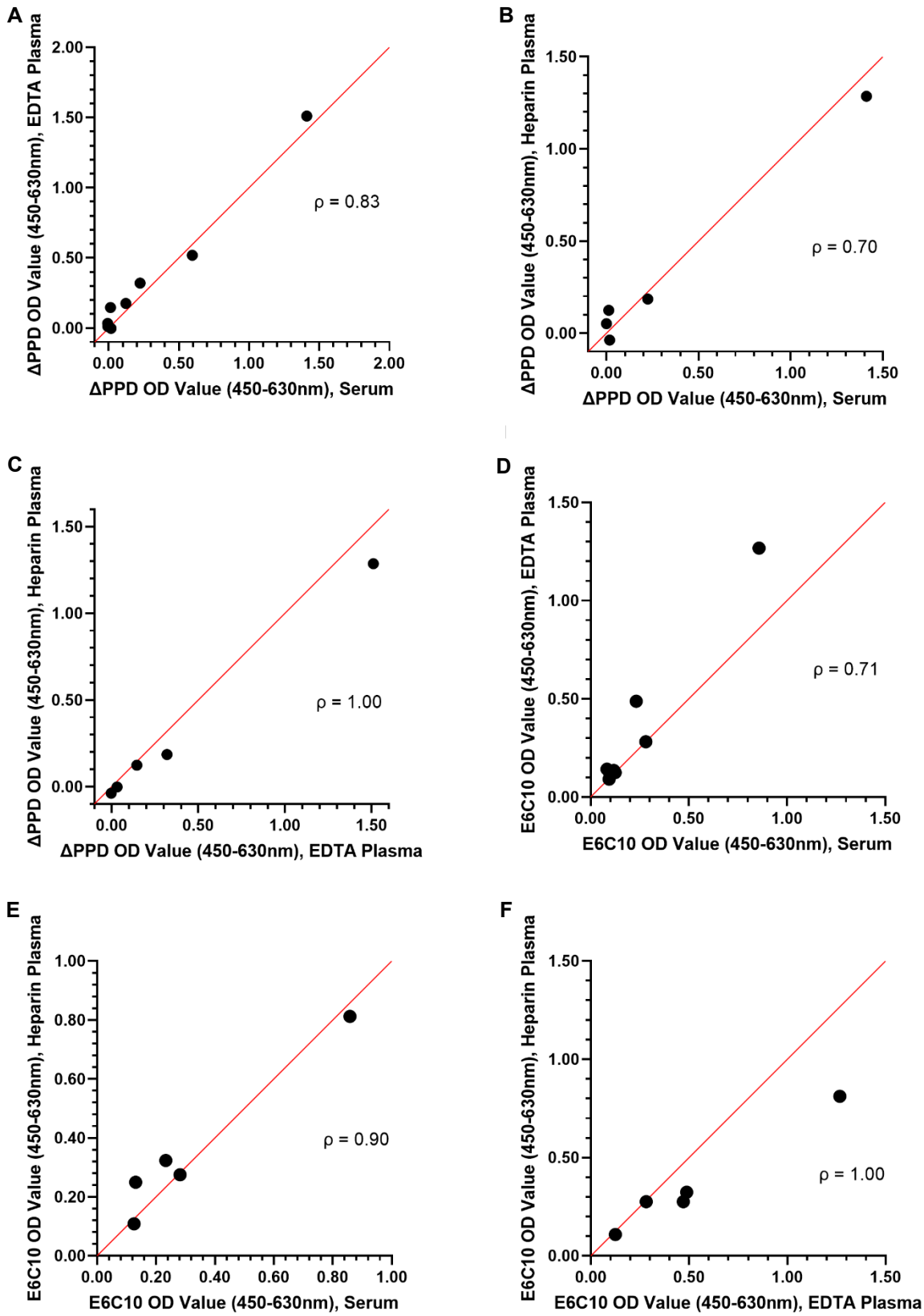


**Figure 6.3:** Correlation analysis across different biological matrices for use in a ΔPPD (A-C) and ESAT-6/CFP-10 (D-F) ELISA for the diagnosis of feline TB. The red line of identity indicates samples where the OD value is identical across both tested matrices. Spearman's rho (ρ) values for each analysis are also reported.

E6C10 = early secreted antigenic target 6kDa/culture-filtrate protein 10kDa (ESAT-6/CFP-10).

| Antigen | Matrix comparison | Spearman's rho | Interpretation | Bias |
|---|---|---|---|---|
| ΔPPD | Serum *vs.* EDTA plasma | 0.83 | Very strong positive | +0.04 (EDTA) |
| | Serum *vs.* heparin plasma | 0.70 | Moderate positive | +0.01 (Serum) |
| | EDTA plasma *vs.* heparin plasma | 1.00 | Perfect positive | +0.09 (EDTA) |
| ESAT-6/CFP-10 | Serum *vs.* EDTA plasma | 0.71 | Moderate positive | +0.09 (EDTA) |
| | Serum *vs.* heparin plasma | 0.90 | Very strong positive | +0.03 (Serum) |
| | EDTA plasma *vs.* heparin plasma | 1.00 | Perfect positive | +0.17 (EDTA) |

**Table 6.1:** Results of Spearman's rho analysis for correlation of OD values across different biological matrices for the ΔPPD and ESAT-6/CFP-10 ELISA, and Bland-Altman analysis to calculate the average bias between OD values for each matrix comparison. Despite perfect correlation between EDTA plasma and heparin plasma samples, the average bias between these samples was greater than for other comparisons with lower Spearman's rho correlation values, with OD values greater for EDTA plasma samples.

### 6.3.3 Determination of ELISA Sensitivity and Specificity

A total of 56 samples (32 culture- or PCR-diagnosed cases of mycobacteriosis, 24 uninfected cats) were tested to determine antigen positive cut-off values and test sensitivity and specificity. For the ΔPPD ELISA, OD values were plotted for each cat according to its corresponding group based on its infection status *i.e.*, MTBC (cats with *M. bovis*, *M. microti*, or an unclassified MTBC infection, n = 27), NTM (*M. avium*, *M. kansasii, M. lepraemurium* and *M. smegmatis*, n = 5) or uninfected (n = 24). Initial analysis of the data showed OD values suggestive of a PPDB-biased response in a number of cats with MTBC infections, with one cat recording an OD value of 2.04 (Figure 6.4 A). There was no evidence of a PPDA-biased response in any of the five NTM-infected cats, which would have generated a negative OD value, and there was no apparent PPD-biased response in any of the uninfected cats. To calculate a cut-off value for ΔPPD positivity, ROC curve analysis was performed using the OD values for the cats with MTBC infections which were plotted against the OD values of the uninfected cats (Figure 6.4 B). The area under the curve (AUC) was 0.67 (95% confidence interval [CI]: 0.52-0.82), suggesting near acceptable discrimination of this ELISA to discriminate between cats with MTBC infections compared to uninfected cats (Hosmer *et al.*, 2013). Selection of a positive cut-off OD value of 0.13 gave test sensitivity of 40.7% (95% CI: 24.5-59.3%) and a specificity of 100% (95% CI: 86.2-100%). With this threshold, none of the NTM-infected cats were positive on the ΔPPD ELISA.

For ESAT-6/CFP-10, data were plotted for OD values from cats infected with RD1 positive species of mycobacteria (*M. bovis* and *M. kansasii*, n = 13), RD1 negative mycobacteria (*M. avium, M. lepraemurium, M. microti* and *M. smegmatis*, n = 9) or those that were uninfected (n = 24) (Figure 6.4 C). There was less discrepancy between positive and uninfected cats compared to the ΔPPD ELISA, although the AUC suggested excellent discrimination of this ELISA (0.84, 95% CI: 0.70-0.99). An antigen positive cut-off OD value of 0.18 was selected from ROC curve analysis, using the OD values from cats infected with RD1 positive mycobacteria against uninfected cats, giving a test sensitivity of 38.5% (95% CI: 17.7-64.5%) and specificity of 100% (95% CI: 85.7-100%) (Figure 6.4 D). However, applying this threshold back to the original set of data showed a positive result in three cats infected with RD1 negative mycobacteria (Figure 6.4 C; red squares). Expanding the dataset of negative controls to include both uninfected cats and those with RD1 negative mycobacterial infections for ROC curve analysis to determine a cut-off value for ESAT-6/CFP-10 ELISA positivity indicated a threshold of 0.24 to maintain specificity of 100% (95% CI: 89.3-100%), but this reduced sensitivity to 15.4% (95% CI: 2.7-42.2%). Using this expanded dataset, the AUC decreased to 0.75 (95% CI: 0.59-0.92), but this still suggested acceptable discrimination. Neither of the two cats deemed positive on the ESAT-6/CFP-10 ELISA (Figure 6.4 C; purple circles) were positive on the ΔPPD ELISA. Due to the lack of sensitivity, ESAT-6/CFP-10 was not included in the ELISA for testing further samples.

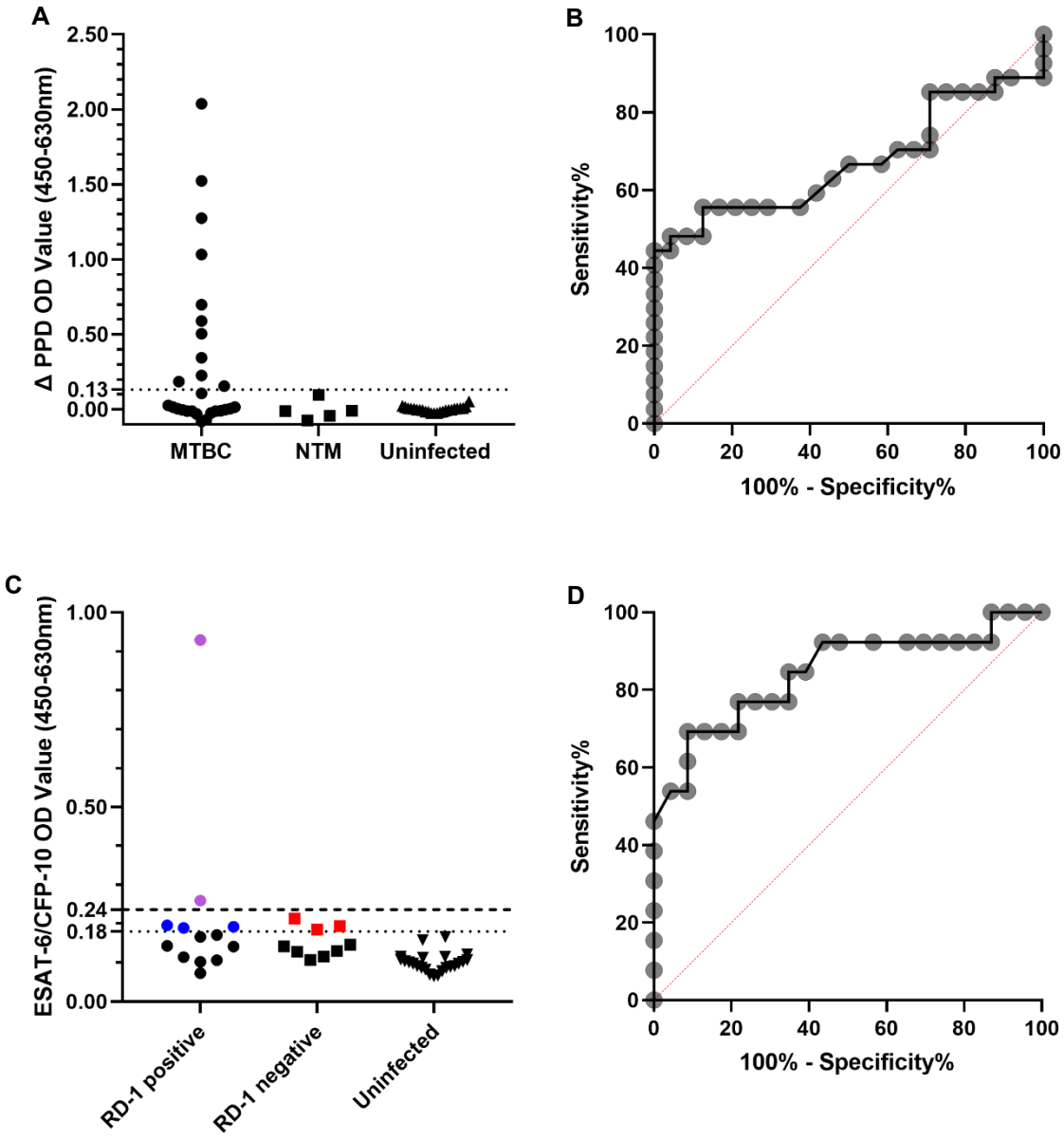


**Figure 6.4:** Results of ELISA testing for the ΔPPD response (A-B) and ESAT-6/CFP-10 (C-D) for cats with culture- or PCR-confirmed TB or NTM infections, and for uninfected 'negative' control cats. A cut-off threshold for ΔPPD positivity of 0.13 (dotted line, A) gave a positive result in 40.7% of cats with MTBC infections; this threshold was calculated from the ROC curve (B) plotted for cats with MTBC infections against uninfected cats. For the ESAT-6/CFP-10 ELISA, an initial cut-off of 0.18 (dotted line, C) calculated from the ROC curve (D) gave 38.5% positivity among RD1 positive mycobacteria (true positives, blue and purple closed circles), but also 33.3% positivity among cats with RD1 negative infections (false positive, red closed squares). A threshold of 0.24 (dashed line, C) was calculated from an expanded 'negative' control group of RD1 negative-infected and uninfected cats; sensitivity reduced to 15.4% (purple closed circles).

## 6.3.4 ELISA Results for Further Cases of Feline Mycobacteriosis

### 6.3.4.1 Samples from Cats with ZN Positive Lesions (Group 1)

Of the samples that came from cats with ZN-positive histopathology or cytology (n = 28), 17 were positive on IGRA (14 showing PPDB-bias and either positive or negative for ESAT-6/CFP-10, two showed PPDA-bias, and one had a PPD-equivalent result [PPDA = PPDB]), five were negative or had a borderline response *i.e.*, positive to one PPD antigen, but lacking PPD-bias), four failed IGRA control thresholds (three due to insufficient stimulation to the positive mitogen control, and one due to both excessive IFNγ production in the unstimulated negative control and a CV > 30% for the ESAT-6/CFP-10 antigen), while two did not have an IGRA test performed. In the antibody tests performed here, a positive ΔPPD response was recorded in 17.6% (3/17) of IGRA-positive cats (Figure 6.5 A); two of these had an IGRA consistent with MTBC infection (PPDB-bias) (OD values = 0.59 and 0.84, respectively) and one with an equivalent PPD-response (OD value = 0.18). The result of this antibody ELISA would suggest that this PPD-equivalent cat had an MTBC infection. A positive ΔPPD response was also recorded in the two cats that were not tested by IGRA (OD values = 0.44 and 1.12, respectively) as well as one cat that was IGRA negative (OD value = 0.83). This cat was also negative on PCR testing for mycobacteria at Leeds University Teaching Hospital on a FFPE tissue sample, but it had consumed the commercial raw food diet associated with the *M. bovis* outbreak and achieved clinical resolution following six months of antimycobacterial treatment with rifampicin, azithromycin and pradofloxacin suggesting it was affected by TB. This cat initially presented with a non-healing lesion on its tail with a mucopurulent discharge, and at physical examination an abdominal mass was discovered in addition to an increase in the respiratory rate and effort (see Figure 1.6).

### 6.3.4.2 Strongly Suspected Cases of Mycobacteriosis (Group 2)

From the cats where mycobacteriosis was strongly suspected (n = 42), 34 had a positive IGRA result, one was negative on IGRA testing, and seven failed control thresholds (three due to insufficient stimulation to the positive mitogen control, two due to excessive IFNγ production in the unstimulated negative control, and two due to CV values exceeding 30%). Of the cats with a positive IGRA result, 29 were suggestive of MTBC infection (PPDB-bias ± ESAT-6/CFP-10 positive), four were suggestive of NTM infection (PPDA-bias, ESAT-6/CFP-10 negative) and one was PPDA-biased, ESAT-6/CFP-10 positive. In total, 38.2% (13/34) of IGRA positive cats were antibody positive on ΔPPD ELISA (Figure 6.5 B); all 13 antibody ELISA-positive cats had an IGRA suggestive of MTBC infection (44.8%, 13/29). In addition, four of the seven cats that had failed IGRA control thresholds were positive on ΔPPD ELISA; this included two cats that failed the positive mitogen control, and one with excessive IFNγ production in the negative control.

### 6.3.4.3 Cases Not Suspected of Mycobacterial Infection (Group 3)

Of the seven cats where mycobacterial infection was not suspected, or an alternative diagnosis was established, five were IGRA negative, one failed the IGRA as the negative control OD value exceeded the threshold, and one gave a PPDA-biased result; this cat was diagnosed with *Actinomyces coleocanis* on PCR at Leeds University Teaching Hospital. The diagnoses in the other cats were idiopathic hypercalcaemia, idiopathic pulmonary fibrosis, pulmonary adenocarcinoma, chronic bronchitis, feline infectious peritonitis, and suspected bacterial bronchopneumonia. All seven were negative on the ΔPPD ELISA (OD value range -0.04 to 0.11), consistent with the lack of a TB diagnosis in these cats.

### 6.3.4.4 Unknown Cases (Group 4)

Cases without sufficient clinical data to accurately categorise their likelihood of being infected with mycobacteria were classified as 'unknown' (n = 68). Twenty-two cats were IGRA positive; 11 had an IGRA suggestive of MTBC infection, seven were suspected of NTM infection and four had a PPD-equivalent result, one of which was also ESAT-6/CFP-10 positive. A negative IGRA result was reported in 34 cats, while five cats failed the IGRA, all due to insufficient stimulation to the positive mitogen control. Seven cats did not have an IGRA performed. A positive ΔPPD result was recorded in 36.4% (8/22) of IGRA-positive cats (Figure 6.5 C); among the positive results, 63.6% (7/11) of cats with an IGRA suggestive of MTBC infection were ELISA positive, while the remaining antibody-positive cat had a PPDA-biased, ESAT-6/CFP-10 IGRA result. Two of the five cats that failed the IGRA were positive on ELISA, as were two of the seven cats without an IGRA result. Among the IGRA negative cats, only 2.9% (1/34) were ELISA positive.

### 6.3.4.5 Testing of In-Contact or Exposed Cats (Group 5)

Samples were available from 41 cats that were overtly healthy with no clinical signs attributable to mycobacterial infection, but either lived in a household with a cat that had been diagnosed with mycobacteriosis (either through culture, PCR, IGRA or positive ZN staining) or had consumed the raw food diet associated with an outbreak of *M. bovis* infection (O'Halloran *et al.*, 2019, O'Halloran *et al.*, 2020). An IGRA result suggestive of MTBC infection was reported in six cats, five of which had consumed the raw food diet (the remaining cat was resident in a multi-cat household where one cat was ZN-positive on histopathology). Three cats had an IGRA result inferring NTM infection, despite having reportedly consumed the raw food diet. Four cats had a PPD-equivalent result, three of which lived in a household with a cat diagnosed with mycobacterial infection and the remaining cat having consumed the raw food diet. Five cats failed the IGRA (three due to insufficient stimulation by the positive control, one due to a high OD value in the negative control, and one due to an excessive CV

value), all of which lived in a household with a cat diagnosed with mycobacterial infection. A negative IGRA result was reported in 23 cats, 12 of which had consumed the raw food diet while the remaining 11 were in-contact with a cat diagnosed with mycobacterial infection.

Only one IGRA positive cat was positive on the ΔPPD ELISA (OD value = 0.70) (Figure 6.5 D); this cat had consumed the raw food diet and lived in a multi-cat household where two other cats were IGRA positive, one of which was also positive for *M. bovis* on culture. This *M. bovis*-culture positive cat was also positive on ΔPPD ELISA (OD value = 0.51), as was the other cat which had lesions suggestive of mycobacterial infection at *post-mortem* examination but was ZN-negative and culture negative (ΔPPD OD value = 1.41). No positive ELISA results were identified among the IGRA negative cats, although one cat did give a strongly negative OD value (ΔPPD OD value = -0.35). This cat lived in a household with an individual that had reportedly been diagnosed with TB, including ZN-positive histopathology on an abdominal mass.

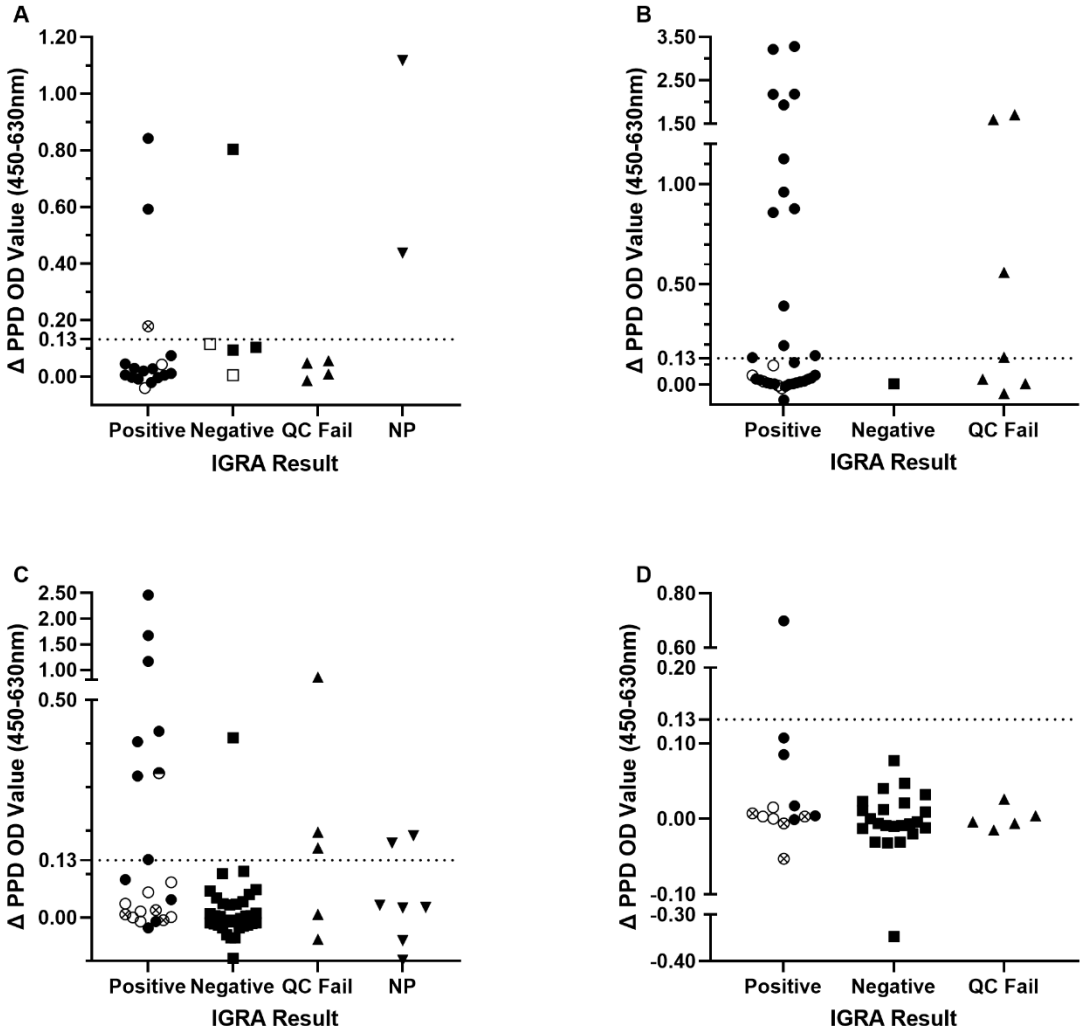


**Figure 6.5:** Results of ΔPPD ELISA testing on cats with ZN positive histopathology or cytology (A), cats where mycobacterial infection was strongly suspected based on clinical signs and response to treatment (B), cases with insufficient clinical data for further classification of the likelihood of mycobacterial infection (C) and cats that had no overt clinical signs consistent with mycobacterial infection and either lived with a cat that had been diagnosed with mycobacteriosis, or had consumed a raw food diet associated with an outbreak of *M. bovis* infection (D). The dotted line at 0.13 indicates the threshold for a positive antibody response.

Closed circles = IGRA suggestive of MTBC infection (PPDB-biased, ESAT-6/CFP-10 positive or negative). Open circles = IGRA suggestive of NTM infection (PPDA-biased, ESAT-6/CFP-10 negative). Open circles with cross = equivalent PPD IGRA response. Half open/half closed circle = PPDA-equivalent, ESAT-6/CFP-10 positive IGRA. Open circle with dot = PPDA-biased, ESAT-6/CFP-10 positive IGRA. Closed squares = negative IGRA. Open squares = borderline IGRA.

QC = quality control. NP = not performed.

## 6.4 Discussion

The data presented in this chapter describes the development and optimisation of a ΔPPD ELISA for the diagnosis of TB in cats, calculates test sensitivity and specificity with the selection of an appropriate cut-off threshold using a well-defined study population, and provides further characterisation of the performance of this test in subsets of field samples from clinical cases. A positive cut-off OD value of 0.13 yielded a test sensitivity of 40.7% and specificity of 100% for the diagnosis of TB in cats with culture- or PCR-confirmed *M. bovis*, *M. microti* or unclassified MTBC infection. No false-positive results were identified in the five cats with culture- or PCR-confirmed NTM infection, and the performance of the ELISA approached acceptable discriminatory power (AUC = 0.67). A positive ELISA result was only reported in 14.3% (2/12) cats that were ZN-positive on histopathology or cytology and had an IGRA result suggestive of MTBC infection; however, a positive ELISA result was also reported in a cat with a PPD-equivalent IGRA result, as well as one cat that was IGRA negative. Test performance was better in cats where mycobacterial infection was strongly suspected, but were ZN-negative or ZN staining was not performed; 44.8% of cats (13/29) that had an IGRA suggesting MTBC infection were antibody-positive, while a further four cats that had failed control thresholds of the IGRA were antibody-positive. A positive ELISA result was reported in 63.6% (7/11) of cats with an IGRA result suggestive of MTBC infection, but without sufficient supporting clinical data to further categorise the likelihood of true infection. Positive results were also reported in one cat with a PPDA-equivalent, ESAT-6/CFP-10 positive IGRA, two out of five cats that had failed the IGRA and 2.9% (1/34) cats that were IGRA negative in this group of 'unknowns'. Among cats that had been IGRA tested as in-contacts or had consumed a commercial raw food diet associated with an outbreak of *M. bovis* infection (O'Halloran *et al.*, 2019, O'Halloran *et al.*, 2020), but did not have overt signs of clinical disease, only one cat was antibody-positive; this cat also had an IGRA result suggestive of MTBC infection.

Attempts to develop and optimise an ELISA using ESAT-6/CFP-10 as the test antigen, which would allow the discrimination between *M. bovis* and *M. microti* infections, were less rewarding. Despite acceptable to excellent discriminatory power (AUC = 0.75-0.84), too many false-positive results were reported in cats infected with RD1 negative species of mycobacteria using a lower antigen-positivity threshold, and at a higher threshold the sensitivity of this ELISA was only 15.4%. Therefore, while the ΔPPD ELISA may not be as sensitive as the IGRA (see Chapter 4) (Mitchell *et al.*, 2021e), or culture for the diagnosis of feline TB (Gunn-Moore *et al.*, 2011a), it does show promise and potential use as an adjunctive diagnostic test to identify cases that may be missed by these, or other, methods.

The format of the ELISA is attractive in terms of its simplicity, repeatability between laboratories, and generally being cost-effective, but there are several steps that require optimisation to maximise performance of the test (Crowther, 2001). The conditions explored for optimisation of the ELISAs performed in this chapter were the antigen dilution or concentration, the choice of antigen coating buffer, and the dilution of Protein A-HRP. Antigen dilution is necessary to minimise background noise and maximise signal, so that positive samples can be readily differentiated from those that are negative (Al-

Adhami and Gajadhar, 2014, Lin *et al.,* 2018). Distinction between positive and negative samples was maintained across all dilutions tested on the ΔPPD ELISA; the decision to use both PPDA and PPDB at 1:2,000 was made given this maintained differential of OD values, and to produce a test with high specificity. Use of PPD antigens at a lower dilution could result in higher OD values in a larger set of uninfected cats, potentially resulting in an unacceptable number of false-positive results. The ESAT-6/CFP-10 dilution series did not demonstrate a decrease in OD values of the singular reactive sample, and the five non-reactive samples also had consistent OD values across the four tested dilution. Therefore, use of ESAT-6/CFP-10 at 5µg/mL for testing further samples was selected as this is the concentration used for stimulation of PBMCs in the IGRA (Rhodes *et al.*, 2008a).

Typically, C-BC buffers have been used as the coating buffer in many ELISAs, including those developed for the diagnosis of TB in cattle (Lyashchenko *et al.*, 1998b, Fontana *et al.*, 2018), but alternative buffers have also been successfully used, such as PBS (Plackett *et al.*, 1989). It has been shown that alternative coating buffers, such as acetate buffer (Cuvelier *et al.*, 1996) or even water (Shrivastav *et al.*, 2003) may give better results compared to C-BC buffers, hence the choice of coating buffer represents an important, yet often overlooked stage in the optimisation of an ELISA. The data presented in this study did not appear to show a major difference between OD values when either C-BC buffer or PBS were used for coating the test plate wells with antigen. Hence, the decision to use C-BC buffer was made on the basis that it is more routinely used for antigen coating in ELISAs than PBS.

Detection of bound matrix antibodies was achieved using HRP conjugated to Protein A. Protein A is derived from *Staphylococcus aureus*, and it binds to the Fc region of various Ig classes and isotypes across a range of species (Goudswaard *et al.*, 1978, Choe *et al.*, 2016). In cats, Protein A binds to IgG and to a lesser extent IgM and possibly IgA (Goudswaard *et al.*, 1978, Lindmark *et al.*, 1983, Strietzel *et al.*, 2014), while Protein G does not bind to feline Ig. Protein A, or a Protein A/G fusion has been used in ELISAs for the diagnosis of toxoplasmosis of naturally and experimentally infected cats, as well as in other species (Al-Adhami and Gajadhar, 2014, Wait *et al.*, 2015). Use of Protein A allows for greater adaptability of the ELISA to other species, but it has been suggested that anti-feline IgG as the detection agent may improve sensitivity under certain conditions (Wait *et al.*, 2015). For this ELISA, Protein A-HRP was used as the detection agent at a range of dilutions to optimise the protocol to maximise test sensitivity while not compromising specificity. Use of Protein A-HRP diluted 1:20,000 yielded OD values that allowed for clear differentiation between reactive, infected cats compared to non-reactive infected and uninfected cats, and as Protein A-HRP was diluted further the distinction between reactive and non-reactive cats became less apparent. Therefore, use of Protein A-HRP diluted 1:20,000 was deemed appropriate for further development of this ELISA.

Another important consideration in the development of a diagnostic test is the type(s) of sample that can be analysed using that platform. Traditionally, serum is used for the detection of antibodies against mycobacterial antigens, and it is sometimes assumed that biomarker presence will be identical regardless of the matrix examined, but this is not always the case. In humans with rheumatoid arthritis it has been shown that detection of IL-24 in EDTA plasma samples gave a higher OD value compared

to matched serum and heparin plasma samples (Kragstrup *et al.*, 2013), whereas measurement of beta-2 microglobulin, a component of major histocompatibility complex (MHC) class I measured as an indicator of cell turnover and renal function, was lower in plasma samples compared to serum (Bjerrum and Birgens, 1986). These differences could result from inherent characteristics of the matrices themselves; plasma contains fibrin, unlike serum, and the polymerisation of fibrin could 'bind' other factors present within the matrix (O'Neal *et al.*, 2014), and platelet activation can result in the release of proinflammatory cytokines which could alter readout values of biomarkers (O'Neal *et al.*, 2014). It is imperative to determine whether differences exist between matrices, because this could impact on the interpretation of a test result being positive or negative, and the potential implications of a misdiagnosis *e.g.*, instigating treatment where it may not be necessary, or missing cases of disease and withholding treatment when it is required. Regarding antimycobacterial antibody testing specifically, one study showed excellent correlation between serum and heparin plasma for the detection of IgG and IgA antibodies by ELISA against malate synthase, MPT51 and arabinomannan, suggesting interchangeability of the two matrices for antibody testing (Siev *et al.*, 2011). Similarly, a recent study showed excellent correlation of antibody titres to MPB70/MPB83 across whole blood, plasma, serum, and diaphragmatic tissue extract using the DPP BovidTB Assay in cattle naturally and experimentally infected with *M. bovis* (Lyashchenko *et al.*, 2021a).

The data presented in this chapter are consistent with what has been observed in these previously reported studies, in that antibody titres correlate well across different blood matrices (Spearman's rho = 0.70-1.00). However, when calculating the average bias in OD values between paired samples, the differences ranged from 0.01 (serum *vs.* heparin plasma, ΔPPD) to 0.17 (EDTA plasma *vs.* heparin plasma, ESAT-6/CFP-10). While the differences may be small, if the cut-off values for determining test positivity are low, these small discrepancies could result in erroneously reporting a result as positive if tested with one matrix *e.g.*, EDTA plasma, whereas if a heparin plasma sample were tested it may be called negative. The differences observed between matrices in this study may result from previous storage conditions of the samples, haemolysis, or binding of antibodies in 'clots' present in the thawed plasma. In veterinary practice, the choice of samples available for testing may be somewhat limited, particularly if only remnant samples can be utilised as was the case in this study; therefore, it is important to determine whether samples can be used interchangeably. These data would suggest there is broadly good correlation of OD values and therefore antibody titres across different blood matrices, but preference should be given to testing of serum and heparin plasma samples over EDTA plasma where available. Further validation of this ELISA could be achieved by testing negative control samples of different matrices to derive matrix-specific cut-off values.

Having selected a final protocol for testing samples, both the ΔPPD and ESAT-6/CFP-10 ELISAs were performed on samples from cats with confirmed mycobacterial infections, known uninfected cats, and assumed healthy, owned cats. This allowed for exploration of test performance on a larger set of samples, as well as establishing antigen-positivity control thresholds and determining performance parameters such as test sensitivity and specificity. Detection of antibodies to PPDB in cattle has been investigated, and a recent meta-analysis suggested a sensitivity of 62%, with a specificity of 96%

(Nuñez-Garcia *et al.*, 2018), but individual studies have shown inconsistent results when testing for anti-PPDB antibodies (Waters *et al.*, 2006a). Anti-PPDB antibodies have been detected in calves as early as three months post-infection (Hanna *et al.*, 1992), but there are concerns of false-positive results in uninfected animals (Amadori *et al.*, 1998), as well as cross-reactions due to infection with environmental mycobacteria such as *M. avium* (Plackett *et al.*, 1989) and *M. kansasii* (Waters *et al.*, 2006b). Therefore, a comparative assay to investigate the presence of anti-PPDA antibodies which could cross-react with PPDB is recommended for the specific diagnosis of TB (Plackett *et al.*, 1989). While the sensitivity of ΔPPD ELISAs is lower than that of PPDB-only ELISA at 44%, there is an increase in specificity to 98% (Nuñez-Garcia *et al.*, 2018).

When the ΔPPD ELISA was performed on samples from cats with culture- or PCR-confirmed diagnoses of mycobacteriosis, responses exceeding OD values reported for the uninfected, negative control cats were only seen in those with MTBC infections; none of the NTM-infected cats showed a PPDA-biased antibody response. While antibodies against PPDA have been identified in *M. avium*-infected cattle (Nielsen *et al.*, 2001, Huda and Jensen, 2003), it is likely the lack of power resulting from a small sample size of NTM-infected cats, specifically those infected with *M. avium*, explains why no PPDA-biased antibody responses were identified. The positive threshold OD value was derived from ROC curve analysis of MTBC-infected cats against the uninfected controls and resulted in an ELISA with reasonable sensitivity for the antibody-based diagnosis of TB (40.7%), excellent specificity (100%), and a test approaching acceptable discriminatory value (AUC = 0.67). One consideration when establishing a positive cut-off value for a diagnostic test is the demographics of the negative control population against which the cut-off value is calculated. For this ELISA, the negative control population predominantly consisted of SPF cats. While SPF animals provide minimal background for the generation of a cut-off value for a positive diagnostic test result, it could result in an 'artificially low' threshold due to the completely immunologically naïve nature of these animals when compared to field samples of healthy, uninfected animals (Moskaluk *et al.*, 2021). The NTM-infected population provided a small group of animals against which the positive cut-off OD value for the diagnosis of TB could be compared; as all five of these cats were negative, it provides further strength to the selection of this OD value as the threshold for ΔPPD ELISA positivity.

Proteomic analysis shows there is a high degree of shared proteins between PPDA and PPDB; therefore, antibody cross-reactivity is likely (Infantes-Lorenzo *et al.*, 2017), suggesting it would be better to use a single-antigen or defined polypeptides for testing. One such polypeptide is the ESAT-6/CFP-10 fusion; these proteins are expressed by members of the MTBC, other than *M. microti* and *M. bovis* BCG (Pym *et al.*, 2002), so this represents an attractive specific-target for *M. bovis*, and in humans, *M. tuberculosis*. However, results of antibody testing for ESAT-6/CFP-10 in other species are mixed. In elephants, ESAT-6 is the dominant antigen for the serological diagnosis of *M. tuberculosis* infection (Lyashchenko *et al.*, 2006), but in other species this is not the case. In humans, some studies have shown that antibody titres to ESAT-6/CFP-10 are higher in those with TB compared to non-infected endemic and non-infected non-endemic controls (Coppola *et al.*, 2017), while other studies have suggested there is poor discriminatory power for ESAT-6/CFP-10 antibody testing between cases of

active TB and exposed controls (Hoff *et al.*, 2007). Many studies of *M. bovis*-infected cattle have shown poor sensitivity for the detection of antibodies against either ESAT-6, CFP-10, or the fusion protein (Waters *et al.*, 2006a, Whelan *et al.*, 2010, Lyashchenko *et al.*, 2017, Waters *et al.*, 2017, Fontana *et al.*, 2018), hence these are not used as primary antigens in antibody-based diagnostics in this species. This also appears to be true for cats, with poor antibody responses to ESAT-6/CFP-10 reported in previous studies (Fenton *et al.*, 2010, Rhodes *et al.*, 2011).

Given the need to differentiate between *M. bovis* and *M. microti* infections, ESAT-6/CFP-10 was used as an ELISA antigen to see if it could be used to successfully discriminate between these two causes of TB in cats. Unlike the ΔPPD ELISA, selection of a positive cut-off value from ROC curve analysis resulted in a high number of false-positive results, this time in cats infected with RD1 negative mycobacteria (*M. microti* and NTM, but not *M. kansasii*), highlighting the previous point of deriving an artificially low cut-off value from SPF cats (Moskaluk *et al.*, 2021). When the negative control group was expanded to include cats infected with RD1 negative species of mycobacteria, a positive test threshold was selected which maintained 100% specificity, but reduced sensitivity to 15.4%. It has been shown that the antigenic composition of mycobacteria changes over time, and that in humans with TB antibodies to ESAT-6 are higher in those with inactive disease rather than active TB (Davidow *et al.*, 2005). Given the samples tested in this study were from cats with active disease undergoing diagnostic investigations, this may provide one reason for the lack of ESAT-6 specific antibody detection in cats infected with *M. bovis*. Additionally, these differences in antigen expression can influence the host immune response, and the antibody profile generated in the face of mycobacterial infection appears to be heterogenous both between and within species (Lyashchenko *et al.*, 1998a, Lyashchenko *et al.*, 2008, Bezos *et al.*, 2014). This lack of sensitivity for the antibody-based diagnosis of *M. bovis* infection is consistent with previous studies in cats (Fenton *et al.*, 2010, Rhodes *et al.*, 2011) and cattle (Fontana *et al.*, 2018), and therefore precludes use of ESAT-6/CFP-10 in ELISA testing.

Having established an appropriate positive cut-off value for the ΔPPD ELISA, groups of field samples collected from cats undergoing diagnostic investigation for suspected mycobacterial infection were tested. In total, 19.9% (37/186) of cats were antibody-positive; however, if considering just those with an IGRA suggestive of MTBC infection, 38.3% (23/60) of cats were positive on ΔPPD ELISA, while there were no positive antibody results in the seven cats that were ultimately diagnosed with something other than mycobacteriosis. The performance of this ELISA on cats with an IGRA indicating MTBC infection is similar to the calculated sensitivity of 40.7% against culture- and PCR-confirmed cases of TB, suggesting validity of this threshold. While the sensitivity of this ELISA is lower than that of IGRA (see Chapter 4) (Mitchell *et al.*, 2021e) or culture (Gunn-Moore *et al.*, 2011a), antibody-testing may still play a role in the diagnosis of feline TB. Among those with ZN-positive histopathology or cytology (Group 1), or where mycobacterial infection was strongly suspected (Group 2), positive antibody-responses were identified in four cats that had failed IGRA control thresholds, and one cat that was IGRA negative. In these cases, it is currently recommended to re-sample the cat to repeat the IGRA (Mitchell *et al.*, 2021e). However, if the positive mitogen control has failed it may indicate a state of anergy (Chappert and Schwartz, 2010), therefore repeat IGRA may also result in test failure; this has

been identified in 16 cats tested to date (J. Mitchell, unpublished data) Given the IGRA is run on heparinised blood, the remnant plasma harvested when processing the sample could be tested in an antibody-ELISA which may allow the diagnosis of TB without requirement for repeat sampling. The identification of antibody-positive ΔPPD responses in cats with a negative IGRA is also important. Previously, it had been suggested that a negative IGRA result in the face of ZN-positive staining indicated infection with a non-*M. avium* NTM species of mycobacteria (O'Halloran and Gunn-Moore, 2017), but it has since been shown that negative IGRA results have also been reported in cats with TB (Chapter 4) (Mitchell *et al.*, 2021e). These negative results, despite an appropriate response to the positive mitogen control, suggests that a state of T-cell anergy has not been induced, but that the circulating memory T-cells present in the sample do not recognise the mycobacterial antigens. This could arise from compartmentalisation of these antigen-specific T-cells to the site(s) of infection (Losi *et al.*, 2007, Jafari *et al.*, 2008), the cat may have been sampled prior to the development of a specific T-cell response (Klenerman *et al.*, 2002), or there may be other factors such as advanced age which has been recognised as a risk factor for false-negative IGRA results in humans with active TB (De Visser *et al.*, 2015, Yamasue *et al.*, 2020). The availability of an antibody test could be useful to diagnose TB in some of these cases.

A diagnosis of TB was also made on the ΔPPD ELISA in one cat with a PPD-equivalent result on IGRA. It has been shown that some cats with culture- or PCR-confirmed MTBC infection can generate PPD-equivalent IGRA results and PPD-equivalent results have also been recorded in some infections with NTM (Černá *et al.*, 2020). Therefore, a PPD-equivalent result cannot determine infection with either MTBC or NTM, so either repeat IGRA testing or alternative diagnostics need to be performed to determine which group of mycobacteria is responsible for the infection. This is important because of differences in treatment protocols and prognosis between these groups of mycobacteria (Gunn-Moore, 2014). As above, the availability of an antibody-based ELISA test, which could be performed on the remnant heparin plasma harvested during processing of the whole blood sample for IGRA, could provide a more rapid means of obtaining a diagnosis of TB and reduce the need for repeat sampling.

While these results are promising for the development of an antibody-based diagnostic test for cases of feline TB, there are further steps that could be investigated. There are many steps within an ELISA that require optimisation, and while the work presented in this chapter addressed some of these, there are further areas that could be tested. These include sample dilution (Crowther, 2001), use of anti-cat Ig for the detection of bound matrix antibodies (Wait *et al.*, 2015), and the effect of different blocking buffers (Vogt Jr *et al.*, 1987). Similarly, there are sample factors that should be explored, namely the effect of haemolysis and freeze-thawing. In a study on the effects of haemolysis and temperature (including sample storage and freeze/thawing) for an ELISA to detect antibodies to *Erysipelothrix rhusiopathiae* in pigs, the OD values were statistically lower in severely haemolysed samples, and this could result in the misclassification of samples on a positive-negative classification if the OD value was close to the threshold in clean samples (Neumann and Bonistalli, 2009). Similarly, the OD values for infectious bronchitis virus and chicken anaemia virus were lowered in haemolysed samples tested by ELISA, whereas the OD values were increased in haemolysed samples that were tested for antibodies

against avian encephalomyelitis virus (Kurian *et al.*, 2012). Haemolysis was not identified as affecting OD values in a suid herpesvirus 1 ELISA for Eurasian wild boar, but repeated freeze-thaw cycles were associated with a decrease in OD values (Boadella and Gortázar, 2011). Repeated freeze-thaw cycles were shown to decrease OD values for the detection of IL-24 in humans with rheumatoid arthritis (Kragstrup *et al.*, 2013), but it was not shown to adversely affect the results of antibody testing for measles, mumps and rubella virus (Pinsky *et al.*, 2003). The samples used in this study were not scored for the degree of haemolysis, nor were samples recorded for the number of freeze-thaw cycles they underwent, so further studies should seek to investigate what impact these factors have on this ΔPPD ELISA.

Finally, other antigens could be tested to determine whether these can provide better test sensitivity, while maintaining specificity, for the diagnosis of feline TB. In cattle, MPB83 and MPB70 have been identified as major immunogenic proteins for the diagnosis of *M. bovis* infection (Cho *et al.*, 2009, McNair *et al.*, 2001, Whelan *et al.*, 2010, Waters *et al.*, 2011b), and a meta-analysis of MPB70 ELISAs for the diagnosis of bTB reported a sensitivity of 70% (Nuñez-Garcia *et al.*, 2018). It has been shown that MPB83 is a major immunogenic protein in other species, including badger and deer (Lyashchenko *et al.*, 2008), and antibody-positive results to this antigen have been reported in cats tested by MAPIA and rapid tests, although the test performance for *M. microti*-infected cats was poor compared to those infected with *M. bovis*, with only 41.7-46.2% of cats with *M. microti* infection positive to tests incorporating MPB83 compared to 90-100% of *M. bovis*-infected cats (Rhodes *et al.*, 2011). It has been reported that *M. kansasii*-infected cattle can give antibody-positive results on MPB83 ELISA (Green *et al.*, 2009) and given this pathogen has been reported as causing disease in cats (Lee *et al.*, 2017, Černá *et al.*, 2020) single-antigen testing for MPB83 alone could result in the misdiagnosis of this infection as TB. Another consideration for the use of MPB83 for antibody-testing is the preparation of this antigen, as recombinant MPB83 may have altered conformation which could result in reduced antibody binding, lowering test sensitivity compared to use of native protein (McNair *et al.*, 2001).

While most antibody-based diagnostics are based on testing blood matrices, another sample that could be tested is stimulated lymphocyte supernatant, which is generated as part of the IGRA methodology (Rhodes *et al.*, 2008a). Antibody in lymphocyte supernatant (ALS) assays have been developed for the diagnosis of TB (Raqib *et al.*, 2003, Raqib *et al.*, 2004, Raqib *et al.*, 2009, Sariko *et al.*, 2017) as well as other infectious diseases such as enteric fever due to *Salmonella* (*S.*) Typhi and *S.* Paratyphi A (Darton *et al.*, 2017). Antibodies in blood matrices such as serum originate from long-lived plasma cells that reside in protected biological niches, such as the bone marrow, allowing for the maintenance of humoral immunity (Tarlinton *et al.*, 2008). Therefore, antibodies in the presence of blood matrices may reflect previous infection and not relate to current, active infection. It has been hypothesised that antigen-specific B-cells *i.e.*, plasmablasts, are short-lived and are only present in the circulation during active disease (Manz *et al.*, 2005). Therefore, isolation and stimulation of these circulating antigenic-specific cells will result in the secretion of antibodies into supernatant, akin to the methodology behind the IGRA. An alternative approach would be to actively isolate antibody-secreting cells from the peripheral circulation, with subsequent detection of these cells in an enzyme-linked immunosorbent

spot (ELISPOT) assay; this has been shown to be a sensitive technique to facilitate the diagnosis of TB (Sousa *et al.*, 2000). It has also been shown that the ALS can be used for the diagnosis of TB in those who are in-contact with individuals with active TB (Raqib *et al.*, 2004), and that a decrease in the OD value corresponds with a reduction in the antigenic burden and therefore indicates successful response to treatment (Raqib *et al.*, 2003, Raqib *et al.*, 2004). This contrasts with the results of serial IGRA testing in cats (Chapter 4) (Mitchell *et al.*, 2021c) and humans (Clifford *et al.*, 2015), where the majority of cats and humans remain IGRA-positive despite achieving clinical resolution of disease. Given this, it would be worthwhile investigating whether antibodies against mycobacterial antigens can be identified in the supernatant that is harvested following the incubation of isolated PBMCs with the mitogen- and antigen-stimulated cells in the feline IGRA, and for cases that underwent serial IGRA testing whether a decrease antibody-specific OD values is also seen. Similarly, the use of ELISPOT to detect circulating antibody-secreting cells to monitor the response to treatment has been shown to be promising, with a reduction in the number of detectable cells over the course of treatment whereas follow-up testing for circulating antigen-specific antibodies by ELISA was of little benefit for this purpose (Sousa *et al.*, 2000). Therefore, a combination of ALS and ELISPOT approaches could be advantageous for providing further insights into the kinetics of the humoral immune response during the treatment of feline mycobacterial infections.

Antibody-based diagnostic tests have traditionally been overlooked for the diagnosis of TB, as the paradigm has been that antibody-positive responses are only detected late in the course of infection, as CMI wanes and the burden of disease progresses (Ritacco *et al.*, 1991). However, recent developments suggest that the humoral immune response may play a more active role in cases of TB than previously thought (Rijnink *et al.*, 2021), and antibody-based diagnostics may show some utility. The work presented in this chapter describes the development of a ΔPPD ELISA for the diagnosis of TB in cats. This test had excellent specificity (100%), with reasonable sensitivity (40.7%); therefore, while a negative result would not rule out TB, a positive result would be highly supportive of a diagnosis of MTBC infection. Further optimisation of the methodology could help improve test sensitivity, so that while this antibody-based test may not provide an alternative for diagnosing cases of TB, it could be a useful adjunctive test for cats that where IGRA testing failed, or a negative IGRA result was reported, and would avoid the need for repeat sampling as well as provide a rapid diagnosis compared to other testing methodologies, such as mycobacterial culture or molecular-based diagnostics.

# Chapter 7: Development of a qPCR Assay for the Diagnosis of Feline Mycobacteriosis

## 7.1 Introduction

While immunodiagnostic tests play a vital role in the identification of cases of mycobacterial infection in veterinary species, namely cattle and cats, they are somewhat limited in that they are an indirect assessment of whether an individual is infected as they do not directly detect the pathogen itself. This can raise concerns surrounding cases with immunological evidence of mycobacterial disease (*i.e.*, a positive IGRA) but no clinical signs consistent with mycobacteriosis as to the specificity of these responses. This immunological response may reflect active disease, latent infection, or even the generation of a memory T-cell response in the face of a cleared infection (Drain *et al.*, 2018). Tests of pathogen detection, however, allow for the direct demonstration of an organism as causing clinical lesions; as such, specialist mycobacterial culture remains the reference standard test for the diagnosis of feline mycobacteriosis in the UK (Middlemiss and Clark, 2018). Despite culture being the reference standard test there are limitations with this approach (see *1.1.4.3.2.1 Mycobacterial Culture*) and as such alternative approaches of pathogen detection are required. Molecular methods, namely PCR and qPCR, provide one such means by which this can be addressed.

Molecular diagnostics have the advantage over mycobacterial culture in that a result can be obtained in a much shorter timeframe, namely days to weeks (O'Halloran *et al.*, 2020) rather than the several months it can take for some species of mycobacteria to be successfully isolated on culture media. For example, it may take a minimum of three months to achieve a culture-confirmed diagnosis of *M. microti* in a case of feline mycobacteriosis (Smith *et al.*, 2009), by which point if the cat had not received appropriate antimycobacterial treatment its health and welfare may be severely compromised to the point that treatment may no longer be considered appropriate, resulting in euthanasia. Additionally, some species of mycobacteria that cannot be successfully cultivated on media can only be diagnosed using molecular approaches (Fyfe *et al.*, 2008, O'Brien *et al.*, 2017a), highlighting the increased range of organisms that can be identified using these approaches compared to traditional culture techniques. A third consideration is the availability of appropriate material for tests of pathogen detection, as culture can only be performed on fresh or frozen tissue samples whereas molecular diagnostics can be performed on both fresh/frozen and formalin-fixed tissue samples (Davies *et al.*, 2006, Reppas *et al.*, 2013, Surat *et al.*, 2014, O'Halloran *et al.*, 2019). This is of particular importance in cases of feline mycobacteriosis because cases may present with singular cutaneous or subcutaneous masses which are completely removed by the PVS and submitted to a diagnostic laboratory entirely fixed in formalin for histopathology on the suspicion of neoplasia. It is only at the time of histopathological examination that mycobacterial infection is suspected, by which point culture can no longer be performed as there are no lesions that can be sampled for the submission of fresh tissue (Gunn-Moore, 2014). Molecular approaches also offer advantages over immunological diagnostics; for example, a positive IGRA result can be reported in cases of feline infection with NTM, but infection with different species of NTM cannot be distinguished (see Chapter 4) (Rhodes *et al.*, 2011, Mitchell *et al.*, 2021e). In contrast, molecular approaches can provide a species-level diagnosis of NTM infections, allowing for the instigation of species-specific treatment protocols.

Currently in the UK, molecular diagnosis of feline mycobacterial infections is provided by the Leeds Teaching Hospitals NHS Trust (The Leeds Teaching Hospitals NHS Trust, 2021). Tests that are optimised and validated for the diagnosis of human mycobacterial infections on samples such as sputum are used (Richter *et al.*, 2003, Richter *et al.*, 2006), but these tests have not been validated for the use on animal samples or on FFPE tissues. Another consideration is that these tests may not be financially viable for some owners, namely where a case of MTBC infection has been identified and further testing is required using an LPA to determine whether infection is due to *M. bovis* or *M. microti* (Richter *et al.*, 2003); this test alone can cost in excess of £500. As this is a human diagnostic laboratory, testing to this level of speciation is not routinely performed given that approximately 99% of cases of human TB in the UK are due to infection with *M. tuberculosis* (de La Rua-Domenech, 2006). A further limitation is that samples from cases of feline mycobacteriosis may have very few mycobacterial organisms present, therefore the concentration of target-specific mycobacterial DNA present within the sample following extraction and amplification may be below the limit of detection of the assay, or there may be insufficient DNA for testing with the LPA following the initial round of PCR for the 16S rRNA gene, which is common in cat samples (O'Halloran *et al.*, 2020, The Leeds Teaching Hospitals NHS Trust, 2021). Despite this, molecular testing for the diagnosis of feline mycobacteriosis in the UK has become more common and is part of the standardised approach to the investigation of such cases (O'Halloran *et al.*, 2019, O'Halloran *et al.*, 2020).

The use of PCR for the diagnosis of feline mycobacteriosis has been investigated for a number of years (Aranaz *et al.*, 1996), with many different approaches used depending on the pathogens of interest. Gene targets commonly investigated in cases of feline mycobacteriosis include the pan-mycobacterial 16S rRNA and *hsp65* genes (Hughes *et al.*, 1997, Malik *et al.*, 2002, Kipar *et al.*, 2003, Hughes *et al.*, 2004, Fyfe *et al.*, 2008, Hetzel *et al.*, 2012, Reppas *et al.*, 2013, O'Brien *et al.*, 2017b); while regions of these genes are highly conserved across species of mycobacteria (Dai *et al.*, 2011), there are hypervariable regions which can be exploited for sequencing, allowing for the species-level identification of mycobacteria in most, but not all cases of infection (Tortoli *et al.*, 2017). Where MTBC infections are suspected, targets such as IS*6110* and *mpb70* have been utilised in PCR and/or qPCR (Aranaz *et al.*, 1996, Peterhans *et al.*, 2020), as these conserved genes are specific to members of the MTBC. However, subsequent differentiation of MTBC organisms can prove to be more challenging; the use of HRM assays for *gyrA and gyrB* SNPs has been trialled in a small number of cats (Peterhans *et al.*, 2020), but typically approaches for the species-level identification of MTBC infection have been based on detecting the presence of RD genes. Genomic analysis of MTBC organisms has proposed an evolutionary scenario for different members of this complex, based on the loss of various RD segments of the genome (Brosch *et al.*, 2002), and patterns of target gene presence from within selected RDs have been used to discriminate between members of the MTBC to the level required of the study (Huard *et al.*, 2003, Pinsky and Banaei, 2008, Halse *et al.*, 2011, Costa *et al.*, 2014).

Given that cases of feline mycobacteriosis may be investigated without initial suspicion of this disease process, lesions may be removed in their entirety and submitted fixed in formalin for histopathology. This limits the availability of fresh tissue for further diagnostics but provides an invaluable bank of FFPE

tissue biopsies from which molecular diagnostics can be performed. However, a major limitation of performing molecular tests on FFPE tissue samples is that DNA extracted from such samples is typically fragmented and shorter in length than what can be obtained from fresh samples, and as such fresh tissues are preferred for molecular testing (Reppas *et al.*, 2013). The process of formalin fixation results in cross-linkage formation between DNA and other biological molecules such as proteins, which need to be reversed for enzyme accessibility to allow for target amplification (Koshiba *et al.*, 1993, Weiss *et al.*, 2011). The type of fixative can also result in fragmentation of DNA (Gilbert *et al.*, 2007), and this damage to the DNA can result in the introduction of mutation artifacts during PCR (Srinivasan *et al.*, 2002). Another consideration is the lack of a standardised approach to the extraction of mycobacterial DNA from FFPE tissues, highlighting the difficulty of working with these tissues for molecular diagnostic test development (Gilbert *et al.*, 2007, Sengüven *et al.*, 2014). Given that FFPE tissues may be the only samples available in some cases of feline mycobacteriosis, having a molecular diagnostic that is optimised for use on this material would be advantageous.

One approach to improve the molecular diagnostic capabilities for feline mycobacterial infections is that of qPCR. Compared to conventional PCR, qPCR offers the advantage that it allows for the detection of target DNA in real time rather than requiring post-amplification processing, and it can also be used on a semi-quantitative basis, providing an estimate of the amount of DNA rather than simply being qualitative (Deepak *et al.*, 2007). Additionally, the target sequence length for qPCR is typically much shorter than that aimed for with conventional PCR; this may be beneficial for use on FFPE tissues given that extracted DNA fragments are often relatively short as described above (Lin *et al.*, 2009, Dietrich *et al.*, 2013).

Therefore, the aim of the work described in this chapter was to design a qPCR assay for the diagnosis of feline mycobacteriosis on FFPE tissue biopsy samples, targeting the major mycobacterial pathogens of importance in cats. To achieve this, novel primer and probe sets were designed against gene targets that would allow for the discrimination of different mycobacterial complexes or species; where appropriate, the literature was consulted, and published primer/probe sets were trialled against target DNA sequences. Methods of mycobacterial DNA extraction from FFPE tissue samples were also compared, to allow for the selection of an appropriate technique for the extraction of DNA from further feline mycobacterial FFPE samples with a corresponding culture- or PCR-confirmed diagnosis once a panel of primer/probe sets had been decided upon. The results of the qPCR were then compared to the culture/PCR-diagnosis to assess test performance of this novel assay.

## 7.2 Materials and Methods

### 7.2.1 Control Materials, Sample Acquisition and Storage

Ethical approval for this study was granted by The University of Edinburgh Veterinary Ethical Review Committee (approval no. 79 14).

Genomic DNA (gDNA) previously extracted from *M. bovis* AF2122/97 (American Type Culture Collection [ATCC] BAA-935) and MAP K10 (ATCC BAA-968) were provided by Dr Conor O'Halloran as well as gDNA extracted from remnant whole blood samples from cats and dogs (O'Halloran, 2019). In addition, *M. tuberculosis* H37Rv (ATCC 25618) gDNA that had been previously obtained from Dr Robin Skuce (Agri-Food & Biosciences Institute) was used. Dr Kirsty Jensen (The Roslin Institute) kindly donated extracted gDNA from *M. bovis* BCG Pasteur 1173P2 and *M. smegmatis* (National Collection of Type Cultures [NCTC] 8159, Cornell 3 ATCC 19420. Genomic DNA from *M. microti* (NCTC 8710, ATCC 19422) was donated by Dr Jason Sawyer and Dr Rowan Morris (APHA). Previously extracted DNA from FFPE tissue biopsies of cats with mycobacterial infections (*M. lepraemurium* n = 1, *M. malmoense* n = 1) was also used as control material (O'Halloran, 2019); *M. malmoense* infection was confirmed by culture on a fresh tissue sample submitted to the APHA by the PVS, while *M. lepraemurium* infection was diagnosed by PCR on the FFPE tissue biopsy sample by Leeds Teaching Hospitals NHS Trust. Further validation of *M. lepraemurium*-specific primers and probes was performed on freshly extracted DNA from the same FFPE tissue biopsy sample as had been previously used, as well as from five additional FFPE biopsies selected from archives held at The Roslin Institute (see *7.2.5 Validation of Primer/Probe Sets on Extracted gDNA*); all these cases were diagnosed by PCR performed at the Leeds Teaching Hospitals NHS Trust. Samples were stored at -20°C until required.

### 7.2.2 Primer and Probe Design and Target Selection

Novel primers and probes for each target were designed using Primer-BLAST (National Center for Biotechnology Information). A panel of targets (Table 7.1) designed to identify the main mycobacterial pathogens of cats in GB *i.e.*, *M. bovis*, *M. microti*, and *M. avium*, with additional targets to identify *M. tuberculosis* given the public health implications of this pathogen, *M. lepraemurium*, and *M. malmoense*. Pan-mycobacteria and mammalian DNA markers were also included to confirm successful DNA extraction from FFPE tissue blocks, and the presence of mycobacteria that would not be detected by the complex- or species-specific markers. In addition to the novel primers and probes tested in this study, published primer/probe sets were also tested as detailed below.

Primers were designed to give a product with an intended length between 60 and 150 nucleotides, an optimum melting temperature ($T_m$) of 60.0°C ± 3°C, a size of 20 ± 5 nucleotides, and guanine-cytosine

(GC) content of 40-80%. The internal probe was designed to be 18-27 nucleotides in length, with an optimal length of 20 nucleotides, $T_m$ 60.0°C ± 3°C, and GC content of 40-80%. Probes were labelled with a 5' 6-carboxyfluoroscein (6FAM) reporter and 3' tetramethylrhodamine (TAM) quencher. All primers and probes were checked for target specificity against publicly available nucleotide reference databases. Primer pairs with the lowest self-complementarity and self 3'-complementarity were then selected from the list of suggested results for further testing.

Primers and probes were ordered from Sigma-Aldrich and reconstituted in PCR grade water (Solis BioDyne, Tartu, Estonia) at 100µM. From this, both forward and reverse primers were made to a 10µM stock solution, and then a mix of both forward and reverse primers at 5µM each was prepared. The probe was then made to a 10µM stock solution, and this was used to prepare 2µM aliquots for use in qPCR.

The 16S rRNA gene was selected as a pan-mycobacterial marker, as this is present in all species of mycobacteria and features highly conserved regions that would be suitable for the detection of DNA from this genus (Rogall *et al.*, 1990, Kirschner *et al.*, 1993, Tortoli *et al.*, 2017, O'Halloran, 2019). Primers and probes were designed against the reference *M. bovis* AF2122/97 and MAP K10 genomes, accession numbers LT708304.1:1474294-1475825 and AE016958.1:2751283-2752815 (Garnier *et al.*, 2003, Li *et al.*, 2005).

For identification of members of the MTBC, a novel primer/probe set for Ag85B (*fbpB*, also known as mpt59 and Rv1886c) was designed against *M. tuberculosis* H37Rv (NC_000962.3:c2135867-2138490), *M. bovis* AF2122/97 (LT708304.1:2129525-2130502) and *M. microti* (EU625397.1) (O'Halloran, 2019). Other targets for MTBC identification using published primer/probe sets were *mpb70* (Lorente-Leal *et al.*, 2019) and IS*6110* (Halse *et al.*, 2010).

To distinguish between members of the MTBC targets across four RD were explored (Pinsky and Banaei, 2008, Halse *et al.*, 2011, Costa *et al.*, 2014, Faksri *et al.*, 2016), namely RD1, RD4, RD9, and RD12. A combination of novel and published primer/probe sets were used for detection of RD1 (Halse *et al.*, 2011, Reddington *et al.*, 2011, Song *et al.*, 2018); novel primer/probes were designed against the ESAT-6 gene *esxA* (also called Rv3875) from *M. bovis* AF2122/97 (LT708304.1:4293341-4293628) and *M. tuberculosis* (NC_000962.3:4352609-5352896) (O'Halloran, 2019). A novel RD4 primer/probe set was designed against Rv1511 (*gmdA)* from *M. microti* OV254 (LR882499.1:1710674-1711696) and *M. tuberculosis* H37Rv (NC_000962.3:1703074-1704096), in addition to a published primer/probe set (Halse *et al.*, 2011). To identify RD9, primer/probes were designed against Rv2074 from *M. tuberculosis* H37Rv (NC_000962.3:2330993-2331406), while a published primer/probe set was used to detect RD12 which is present in *M. tuberculosis* and *M. microti* (Halse *et al.*, 2011).

Detection of *M. avium* was accomplished by targeting the specific marker IS*1311* (U16276.1) (O'Halloran, 2019), and the variable region of the 16S rRNA gene (X52918.1). Targets for identification of *M. lepraemurium* were the species-specific genes MLM_1327 and MLM_3300

(CP021238.1:1308101-1308400 and CP021238.1:3341401-3342500, respectively) (Benjak *et al.*, 2017), as well as variable regions of the *sodA* (D13288.1) (O'Brien *et al.*, 2017c) and *hsp65* genes (AY550232.1). Primers/probes designed against the 16S-23S rRNA ITS (Z35225.1) and MA2/6 fragment (AF085175.1) (Glennon *et al.*, 1996, Kauppinen *et al.*, 1999) to detect *M. malmoense*.

Finally, a primer/probe set against the *Felis catus* 18S rRNA gene (NC_058381.1:c13028091-13026225) was designed to act as an endogenous control for qPCR. For potential future application of this assay in other companion animal species, namely the dog, the primers/probes designed against the feline 18S rRNA gene were also designed to be compatible with canine 18S rRNA (XR_005382291.1).

| Name | Target | Forward Primer | Reverse Primer | Probe | Length (bp) | Species/Group | Source |
|---|---|---|---|---|---|---|---|
| 16S rRNA | 16S rRNA | AATTCCTGGTGTAGCGGTGG | GTTTACGGCGTGGACTACCA | [6FAM]-AGGAGGAACACCGGTGGCGA-[TAM] | 143 | Pan-mycobacteria | This study |
| Ag85B | Rv1886c (*fbpB*) | TCGCTGGTCAGGAAGGTTTC | TACCAGTCGGGACTGTCGAT | [6FAM]-TTACCGCAGGCCGGGCTGTA-[TAM] | 128 | MTBC | This study |
| *mpb70* | *mpb70* | CTCAATCCGCAAGTAAACC | TCAGCAGTGACGAATTGG | [6FAM]-CTCAACAGCGGTCAGTACACGGT-[TAM] | 133 | MTBC | Lorente-Leal *et al.*, 2019 |
| IS*6110* | IS*6110* | GGGTAGCAGACCTCACCTATG | AGCGTAGGCGTAGGTGA | [6FAM]-TCGCCTACGTGGCCTTT-[TAM] | 74 | MTBC | Halse *et al.*, 2010) |
| RD-1 | Rv3875 (*esxA*), RD1 | ACCAGGGTGTCCAGCAAAAA | ATCCCAGTGACGTTGCCTTC | [6FAM]-CAGCGAAGCCGGTCAGGCAA-[TAM] | 127 | *M. bovis* *M. tuberculosis* | This study |
| RD-1 TB | Rv3876 (*esxI*), RD1 | CCCTTTCTCGTGTTTATACGTTTGA | GCCATATCGTCCGGAGCTT | [6FAM]-CACTCTGAGAGGTTGTCA-[TAM] | 109 | *M. bovis* *M. tuberculosis* | Halse *et al.*, 2011 |
| RD-1 A | Rv3875 (*esxA*), RD1 | CGTCCATTCATTCCCTCCTT | TACGCCTCCGAACCGCTA | [6FAM]-AAGCAGTCCCTGACCAAGCTCGCA-[TAM] | 85 | *M. bovis* *M. tuberculosis* | Song *et al.*, 2018 |
| RD-1 B | Rv3876 (*esxI*), RD1 | CATCGCTGATGTGCTTGC | TGCGCCGAGCTGTATTC | [6FAM]-ACACTAGCGTCAATGCGGTCA-[TAM] | 117 | *M. bovis* *M. tuberculosis* | Reddington *et al.*, 2011 |
| RD-1 C | Rv3875 (*esxA*), RD1 | ACCAGGGTGTCCAGCAAAAA | ATCCCAGTGACGTTGCCTTC | [6FAM]-CAGCGAAGCCGGTCAGGCAA-[TAM] | 127 | *M. bovis* *M. tuberculosis* | This study |
| RD-4 | Rv1511 (*gmdA*), RD4 | GGCCAAGGGGTATGAGGTTC | CATAGTGCAGAAACAGCCGC | [6FAM]-GGCGCGCTTCGACGTTCAAC-[TAM] | 119 | *M. microti* *M. tuberculosis* | This study |
| RD-4 TB | Rv1508a, RD4 | CCACGACTATGACTAGGACAGCAA | AAGAACTATCAATCGGGCAAGATC | [6FAM]-ACCAGTGAGGAAACC-[TAM] | 87 | *M. microti* *M. tuberculosis* | Halse *et al.*, 2011 |
| RD-9 | Rv2074, RD9 | CCTTCGACCCCAAGACTCAC | TACCCTCCAGTGAGAGCCAG | [6FAM]-AAGGCCGTCAATGCCGACCG-[TAM] | 132 | *M. tuberculosis* | This study |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| RD-12 | Rv3120, RD12 | CGTTGGAACGCGAAATACG | CCAGGATATGGGCGCAAAT | [6FAM]-TGCGCTGACCCCAC-[TAM] | 63 | *M. microti* *M. tuberculosis* | Halse *et al.*, 2011 |
| IS*1311* | IS*1311* | AGAGCAACTCGACACCATCG | AGTGCAGGACAGGGAAATCG | [6FAM]-GAGGCCGCCGACGACATCAC-[TAM] | 116 | *M. avium* | This study |
| MAC1 | 16S rRNA | GCGTCTTGAGGTCCTATCCG | GGCGTGCTTAACACATGCAA | [6FAM]-TGCAGGGCAGATTGCCCACG-[TAM] | 148 | *M. avium* | This study |
| Lep1 | MLM_3300 | CCAGTCTGGCGAATACCAGG | CGTCCCCCAGTTGAGTATCG | [6FAM]-CGATCGCTGACCTCGCGGTC-[TAM] | 134 | *M. lepraemurium* | This study |
| Lep2 | MLM_1327 | TCACCCTCCCCGACAAGATT | ATGTCCCGCGAAACTCAACA | [6FAM]-AGCCGTCTACATGCGCCGTG-[TAM] | 119 | *M. lepraemurium* | This study |
| Lep3 | MLM_3300 | ACACGGGTTCGTACTTCGAC | CTTTCGACTATCGGGGCAGG | [6FAM]-CACGGCTTATCGACAGGGCGA-[TAM] | 67 | *M. lepraemurium* | This study |
| Lep4 | *sodA* | AAAGAACCTCGCCTTCCACC | ACTTGTCGAAGGACCCGAAC | [6FAM]-GCGGCCACGTCAACCACTCA-[TAM] | 137 | *M. lepraemurium* | This study |
| Lep5 | *hsp65* | GGCGCAGGCATTAGTCAAAG | CAGCCTTTTCGATGTCACGC | [6FAM]-AAGGCCTCCGCAACGTCGTG-[TAM] | 86 | *M. lepraemurium* | This study |
| Lep6 | *hsp65* | GCCGAGCTGGTTAAGGAAGT | ACGGTGGCTGTTGTAGTACC | [6FAM]-CCGATGACGTCGCCGGTGAC-[TAM] | 71 | *M. lepraemurium* | This study |
| Mal1 | 16S-23S rRNA ITS | ATTGGGCCCTGAGACAACAC | GAATACGCGTCTACGGGCAG | [6FAM]-CCCCCATCTTGGTGGTGGGGT-[TAM] | 130 | *M. malmoense* | This study |
| Mal2 | MA2/6 | CCGGCGTCGAATATTGCTTG | AGTACTTGCTCAACCCGCTC | [6FAM]-GGATAGCCGCGGCGTACGAG-[TAM] | 108 | *M. malmoense* | This study |
| Mal3 | MA2/6 | CTTGATGCTGTGCGGTGAAG | CCTAACTGGGCTGATTCCCG | [6FAM]-TGCGAATCGCACAGTCAGCGA-[TAM] | 70 | *M. malmoense* | This study |
| 18S rRNA | 18S rRNA | CCCCTCGATGCTCTTAGCTG | GAACCGCGGTCCTATTCCAT | [6FAM]-TTCAAAGCAGGCCCGAGCCG-[TAM] | 129 | Mammalian DNA | This study |

**Table 7.1:** Details of the primers and probes tested as part of development of a qPCR assay for the diagnosis of feline mycobacteriosis on FFPE tissue biopsy samples.

## 7.2.3 Extraction of gDNA from FFPE Tissue Biopsy Samples

Four methods to extract gDNA from FFPE tissues were trialled (Fyfe *et al.*, 2008, Reppas *et al.*, 2013, Surat *et al.*, 2014, O'Halloran, 2019, Lavender and Fyfe, 2022). For all methods, FFPE tissues were trimmed in until the entire surface area of the tissue was exposed, and six sections 10 microns thick were cut using a MB35 Premier microtome blade (Thermo Fisher Scientific) mounted onto a HM325 rotary microtome (Thermo Fisher Scientific) and placed into a sterile 1.5mL screw-top Eppendorf tube. A fresh blade was used for each FFPE tissue.

*Method one:* 320µL of Deparaffinization Solution (Qiagen, Hilden, Germany) was added to each sample, vortexed for 10 seconds, and then incubated at 56°C for three minutes. Following this, the tissue was resuspended in 180µL of Buffer ATL (Qiagen), vortexed for 10 seconds, returned to the heat block at 56°C and 20µL proteinase K (Qiagen) was added to the aqueous phase and mixed by gentle pipetting. After one hour, an additional 10µL of proteinase K was added to samples that were not completely lysed and mixed by gentle pipetting; samples were left for one further hour at 56°C. Samples were then incubated at 90°C for one hour to reverse formalin modification of nucleic acids; following this, the sample was briefly centrifuged to remove droplets from inside the lid, and the aqueous phase was transferred to a clean Eppendorf. To this, 200µL of Buffer AL (Qiagen) was added and then vortexed for 10 seconds before the addition of 200µL of 100% ethanol (Fisher Chemical, Hampton, New Hampshire, USA) and this was vortexed for 10 seconds; this was briefly centrifuged to remove droplets from inside the lid. This was transferred to a DNeasy® Mini Spin Column (Qiagen), centrifuged at 6,000 x *g* for 60 seconds and the flow-through and collection tube discarded. Next, 500µL of Buffer AW1 (Qiagen) was added to the column, followed by centrifugation at 6,000 x *g* for 60 seconds and the flow-through and collection tube discarded. Then, 500µL of Buffer AW2 (Qiagen) was added, centrifuged at 6,000 x *g* for 60 seconds, the flow-through and collection tube discarded, and then the sample was centrifuged again at 16,100 x *g* for three minutes to dry the spin column membrane. The column was placed in a sterile Eppendorf, 50µL of Buffer ATE (Qiagen) was added directly to the membrane and left to incubate at RT for five minutes, and then centrifuged at 16,100 x *g* for 60 seconds to elute the DNA. The concentration and quality of DNA was determined using a Nanodrop-1000 (Thermo Fisher Scientific), and the sample stored at -20°C prior to testing. This is an adaptation of the method used by Surat *et al.* (2014).

*Method two:* to each sample, 1mL of xylene (Genta Medical, York, UK) was added and then centrifuged at 13,000 x *g* for five minutes. The xylene was removed, taking care not to disrupt the tissue pellet; this deparaffinisation step with xylene was repeated. Tissues were rehydrated with 1mL of 100% ethanol, centrifuged at 13,000 x *g* for five minutes, the supernatant carefully removed, and this step was also repeated. Following the removal of ethanol after the second wash, the tubes were left to sit at RT to allow any residual ethanol to evaporate. The sample was incubated in digestion buffer (50mM Tris-hydrochloride [HCl], 10mM EDTA, 0.5% weight/volume [w/v] sodium lauryl sulfate [SLS] [Fisher Chemical], 50mM sodium chloride [NaCl] [Fisher Chemical] and 300µg/mL proteinase K (Qiagen) at

56°C for 24 hours. Following this, samples were incubated at 90°C for 15 minutes. Method one was then followed from the addition of 200µL of Buffer AL, with the exception that Buffer AE (Qiagen) was used to elute DNA rather than Buffer ATE. This protocol was adapted from that by Fyfe *et al.* (2008) and Reppas *et al.* (2013).

*Method three:* each sample was incubated in 1mL xylene at 56°C for 15 minutes, centrifuged at 6,000 x *g* for one minute, the xylene discarded, and this step repeated. Then, the tissue was rehydrated in 100% ethanol, vortexed for 10 seconds, incubated at RT for 15 minutes, centrifuged at 6,000 x *g* for one minute, the ethanol discarded, and this step was repeated once with 100% ethanol, and then twice with 70% ethanol (Fisher Chemical). The sample was rehydrated in 300µL enzymatic digestion buffer (50mM Tris-HCl, 10mM EDTA, 0.5% w/v SLS, and 50mM NaCl) with 20mg/mL lysozyme (Sigma-Aldrich) added immediately before use; this was incubated at 37°C for a minimum of 48 hours. Then, proteinase K was added to a final concentration of 300µg/mL and incubated for one hour at 56°C, before incubation at 95°C for 10 minutes. The sample and lysis buffer were transferred to a tube pre-filled with 0.1mm sterile acid-washed silica beads (Benchmark Scientific, Sayreville, New Jersey, USA) and 180µL Buffer ATL added, and the sample was homogenised at 6.0 metres/second for 40 seconds using a Thermo FastPrep FP120 Cell Disrupter (Thermo Fisher Scientific). The samples were returned to RT on ice, then 200µL Buffer AL was added; the protocol then followed that of method one, other than following the addition of 500µL Buffer AW2 the column was centrifuged at 8,000 x *g* for two minutes and then eluted in 50µL Buffer AE by centrifugation at 8,000 x *g* for one minute. This method was adapted from that used by O'Halloran (2019).

*Method four:* 1mL xylene was added to each sample and left to incubate at RT for five minutes before centrifugation at 16,100 x *g* for five minutes to pellet the tissue. The supernatant was removed, avoiding the cell pellet, and the xylene deparaffinisation step was repeated. Then, the tissue was re-suspended in 1mL 100% ethanol, incubated for five minutes at RT, centrifuged at 16,100 x *g* for five minutes, the supernatant discarded, and the ethanol rehydration step repeated. After this, the pellet was allowed to air dry at RT before being re-suspended in 180µL Buffer ATL; to this, 20µL proteinase K was added and the sample incubated at 56°C for 24 hours. The sample was removed from the heat block, left to cool, and then briefly centrifuged to remove any liquid from inside the lid of the tube. To this, 200µL Buffer AL was added and vortexed for 10 seconds, before incubating at 70°C for 10 minutes, centrifuged briefly to remove liquid from inside the lids, and then 200µL 100% ethanol added to the lysate and vortexed for 10 seconds. The sample was transferred to a spin column and from this point method one was followed, with the exception that after Buffer AW2 was added the column was centrifuged at 16,100 x *g* for three minutes, and the DNA was eluted in 50µL Buffer AE following centrifugation at 6,000 x *g* for one minute. This protocol was adapted from Lavender and Fyfe (2022).

## 7.2.4 qPCR Protocol

For each qPCR reaction, a total master mix volume of 17.5µL was prepared (10µL Precision®Plus 2X qPCR MasterMix with LR [Primerdesign, Southampton, UK], 5.3µL PCR grade water), 1.2µL primer [10µM forward and reverse mix], and 1.0µL 2µM probe). The master mix was briefly vortexed and spun before adding to the PCR tube. To this, 2.5µL of control or sample was added to each reaction master mix, to give a final reaction volume of 20µL; all samples and controls were mixed by pipetting at least three times before addition to the first master mix, and after addition to all master mixes. The PCR tubes were spun down using a Mini Plate Spinner MPS1000 (Labnet International, Edison, New Jersey, USA) to remove any large bubbles and the tubes were then loaded into the QuantStudio™ 5 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). Cycling parameters were set to one cycle at 95°C for two minutes to activate the hotstart *Taq* polymerase enzyme, followed by 40 cycles at 95°C for 10 seconds to denature the DNA, and then 60°C for 60 seconds for primer and probe binding, and elongation of the target DNA sequence. Data for individual reactions were collected from each cycle during the binding and elongation phase and the resulting file saved for visualisation using the QuantStudio™ Design & Analysis Software v.1.5.2 (Applied Biosystems, Thermo Fisher Scientific).

## 7.2.5 Validation of Primer/Probe Sets on Extracted gDNA

Panels of primers/probes were tested against gDNA extracted from mycobacterial cultures (*M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. microti*, MAP, and *M. smegmatis,* n = 1 for each), or from FFPE tissue biopsies of cases of feline mycobacteriosis (*M. lepraemurium* and *M. malmoense,* n = 1 for both); primers/probes were also tested against PCR grade water as a negative control. Primers/probes that showed specific binding to their target DNA sequences across the respective control samples were chosen for further validation against DNA extracted from feline mycobacterial FFPE tissue samples.

To compare DNA extraction methodologies, two feline mycobacterial FFPE tissues (*M. bovis* n = 1, *M. microti* n = 1, both diagnosed by specialist mycobacterial culture) were selected from an archive of tissue biopsy samples held at The Roslin Institute, in addition to a FFPE tissue sample from a cow experimentally infected with *M. bovis*, kindly provided by Professor Jayne Hope. A subset of primer/probes that had shown appropriate binding to DNA from mycobacterial cultures were selected for qPCR; extracted gDNA from *M. microti* and PCR grade water were included as a positive and negative control, respectively. The magnitude of the fluorescence signal (ΔRn) for all targets in both positive and negative controls were examined to select an appropriate cut-off value, which were then used to determine cycle threshold ($C_t$) values for each target; these $C_t$ values were compared for all three samples across each DNA extraction method to determine which method should be selected for further validation of primers/probes against feline mycobacterial FFPE biopsy samples.

For further validation of primer/probes designed to detect *M. lepraemurium*, DNA was extracted from the FFPE tissue block that had been previously used for primer/probe testing (Case 32, Table 7.7), in addition to five other FFPE tissue samples held in tissue archives at The Roslin Institute. These six FFPE tissue biopsies came from a total of four cats *i.e.*, two cats had two FFPE tissue biopsies available. The six primer/probe sets targeting *M. lepraemurium* (Lep1-Lep6) were tested in addition to the pan-mycobacterial 16S rRNA and mammalian 18S rRNA primer/probes on all six extracted gDNA samples and PCR grade water as a negative control.

A final panel of eight primer/probe sets were selected based on their performance against gDNA from mycobacterial cultures or FFPE tissue samples for testing against a larger set of feline mycobacterial FFPE tissue biopsies with a culture- or PCR-confirmed diagnosis. The final study population consisted of tissues from cats infected with *M. bovis* (n = 10), *M. microti* (n = 9), unspecified MTBC (n = 6), *M. avium* (n = 6), *M. lepraemurium* (n = 4), *M. interjectum* (n = 1), *M. kumamotonense* (n = 1), and *M. malmoense* (n = 1). One FFPE tissue sample was selected for each individual cat, and DNA was extracted using the finalised methodology (see *7.3.2 Comparison of DNA Extraction Methods on Bovine and Feline FFPE Samples*); positive control gDNA from *M. tuberculosis*, *M. bovis*, *M. microti*, MAP*,* and *M. smegmatis* was included, along with PCR grade water as a negative control for qPCR. Control sample curves were examined to select ΔRn values from which $C_t$ values could be calculated for the test samples, identifying if samples were positive or negative for each target. The panel of results were then interpreted to assign a species/complex-level infection which could be compared to known culture- or PCR-confirmed diagnosis to determine qPCR sensitivity and accuracy.

## 7.3 Results

### 7.3.1 Results of qPCR on Control gDNA Samples

For the pan-mycobacterial 16S rRNA primer/probe set (16S rRNA), curves were seen in all control gDNA samples from mycobacterial cultures (*M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. microti*, MAP, and *M. smegmatis*, data not shown), and from feline mycobacterial FFPE tissue biopsy samples (*M. lepraemurium* and *M. malmoense,* data not shown for *M. malmoense*). For the negative control samples (PCR grade water, feline gDNA, and canine gDNA) an increase in the fluorescence signal was detected, but selection of an appropriate ΔRn value could clearly differentiate between mycobacterial positive and negative samples. Similarly, for the mammalian 18S rRNA primer/probe (18S rRNA) a late increase in the fluorescent signal was reported in all mycobacterial culture gDNA samples and the PCR grade water negative control, but the increase in fluorescence did not resemble the curves seen in the mammalian gDNA control samples, or the two feline mycobacterial FFPE biopsy samples. These results suggested that the 16S rRNA and 18S rRNA primer/probes were appropriate for testing on further samples, but

negative controls for these markers should be included to select an appropriate ΔRn threshold for calculating $C_t$ values in test samples.

Three MTBC-specific markers were tested: Ag85B, *mpb70*, and IS*6110*. A ΔRn value of 20,000 was selected for all three markers, and the $C_t$ values determined (Table 7.2). For Ag85B and IS*6110*, all four MTBC controls were positive, while the four NTM samples (MAP, *M. smegmatis*, *M. lepraemurium,* and *M. malmoense*) and the water control were negative. The lowest $C_t$ values for all four MTBC samples were seen with detection of IS*6110*, indicating greater amounts of amplified target nucleic material, followed by Ag85B. Only *M. tuberculosis* was positive for *mpb70*.

| **Species** | **IS*6110*** | **Ag85B** | ***mpb70*** |
|---|---|---|---|
| *M. tuberculosis* | 19.5 | 24.2 | 23.9 |
| *M. bovis* | 29.2 | 31.5 | **-** |
| *M. bovis* BCG | 29.1 | 30.2 | **-** |
| *M. microti* | 26.4 | 38.0 | **-** |
| MAP | - | - | - |
| *M. smegmatis* | - | - | - |
| *M. lepraemurium* | - | - | - |
| *M. malmoense* | - | - | - |
| Water | NP | - | - |

**Table 7.2**: Comparison of $C_t$ values for three different qPCR primer/probe sets against different targets specific to the MTBC. All four MTBC samples were positive to the primer/probe against IS*6110* and Ag85B, but only *M. tuberculosis* was positive using a primer/probe set designed against *mpb70*. - = negative. False negative results are highlighted in red and boldface type.

To identify members of the MTBC, different RD markers were explored. For the detection of RD1, five primer/probe sets were tested. As for the MTBC markers, a ΔRn value of 20,000 was selected for determining $C_t$ values (Table 7.3). All five primer/probe sets gave a positive result when tested against *M. tuberculosis*, with similar $C_t$ values ranging from 22.0 for RD-1 A (Song *et al.*, 2018) to 26.8 for RD-1 TB (Halse *et al.*, 2011), but no positive results were recorded for the detection of RD1 in *M. bovis*. In contrast, *M. microti* tested positive for RD1 DNA with all five primer/probe sets. The final MTBC pathogen tested, *M. bovis* BCG, was negative for all five primer/probe sets, and no positive responses were identified from the NTM or water samples where tested.

Other RD markers investigated for the species-level identification of MTBC pathogens were RD4, RD9 and RD12. Two RD4 primer/probe sets were trialled (RD-4 and RD-4 TB), with ΔRn values of 10,000 and 20,000 chosen, respectively. For the RD9 marker (RD-9), a threshold of 40,000 was selected and for RD12 (RD-12) the threshold was set to 20,000 (Table 7.4). For RD4, *M. tuberculosis* was positive with both primer/probe sets tested, whereas *M. microti* was negative to both. All other samples were negative as appropriate, where tested. For RD-9, a positive response was reported only for *M.*

*tuberculosis*; all other samples were RD-9 negative as expected. Finally, both *M. tuberculosis* and *M. microti* were RD-12 positive and all other samples tested were negative, showing an appropriate pattern of responses to this primer/probe set.

| Species | RD-1 | RD-1 TB | RD-1 A | RD-1 B | RD-1 C |
|---|---|---|---|---|---|
| *M. tuberculosis* | 24.5 | 26.8 | 22.0 | 23.3 | 24.2 |
| *M. bovis* | - | - | - | - | - |
| *M. bovis* BCG | - | - | - | - | - |
| *M. microti* | 34.0 | 35.8 | 29.5 | 30.9 | 32.2 |
| MAP | - | - | - | - | - |
| *M. smegmatis* | - | - | - | - | - |
| *M. lepraemurium* | - | - | NP | NP | NP |
| *M. malmoense* | - | - | NP | NP | NP |
| Water | - | NP | - | - | - |

**Table 7.3**: Comparison of $C_t$ values for five primer/probes targeting different portions of the genome called RD1, which is present in *M. tuberculosis* and *M. bovis*, but absent from *M. microti* and *M. bovis* BCG. While all five primer/probe sets gave a positive result when tested against *M. tuberculosis*, there were no positive results when tested against the *M. bovis* gDNA sample. In contrast, *M. microti* was positive with all five primer/probe sets. Unexpected positive results are highlighted in blue. False negative results are highlighted in red/bold.

| Species | RD-4 | RD-4 TB | RD-9 | RD-12 |
|---|---|---|---|---|
| *M. tuberculosis* | 29.7 | 33.9 | 27.7 | 22.5 |
| *M. bovis* | - | - | - | - |
| *M. bovis* BCG | - | - | - | - |
| *M. microti* | - | - | - | 28.9 |
| MAP | - | - | - | - |
| *M. smegmatis* | - | - | - | - |
| *M. lepraemurium* | - | - | - | - |
| *M. malmoense* | - | - | - | - |
| Water | - | - | - | - |

**Table 7.4**: Results of qPCR testing for three further RD portions of the mycobacterial genome. Both RD4 primer/probes (RD-4 and RD-4 TB) were positive against *M. tuberculosis* but were negative against *M. microti*. The RD12 primer/probe (RD-12) was positive for both *M. tuberculosis* and *M. microti* as predicted from known sequence data of both pathogens. Only *M. tuberculosis* was RD-9 positive, and this result was appropriate. False negatives are highlighted in red/bold.

For the identification of *M. avium*, an IS*1311* primer/probe (IS*1311*) and a 16S rRNA primer/probe (MAC1) set were tested. While both primer/probe sets were positive when tested against *M. avium* gDNA, IS*1311* showed greater specificity than MAC1, but MAC1 reached a higher curve plateau. There was no increase in fluorescence from the baseline for all control samples tested other than *M. avium* with the IS*1311* primer/probe, whereas a ΔRn threshold of 40,000 was required to eliminate any false-positive responses to MAC1 in non-*M. avium* samples.

Three primer/probe sets specific to *M. malmoense* were tested: Mal1, Mal2 and Mal3. For all three primer/probe sets no increase in fluorescence beyond the baseline was detected in the non-*M. malmoense* gDNA samples. All three primer/probe sets showed an increase in fluorescence, but the curve for Mal1 only consisted of the exponential-log phase, whereas the curve morphology for both Mal2 and Mal3 appeared more appropriate for specific positive detection of the target DNA. After 40 cycles, the ΔRn value for Mal2 was nearly 40,000 compared to 140,000 for Mal3 (Figure 7.1).

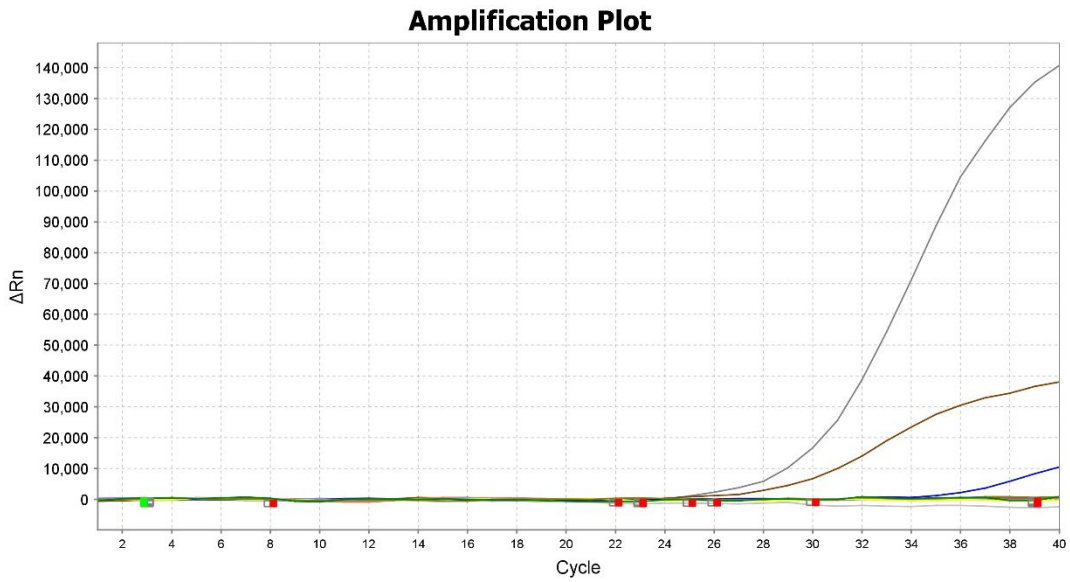


**Figure 7.1:** Results of qPCR on DNA previously extracted from a FFPE tissue sample from a cat with culture-confirmed *M. malmoense* infection. There is a marked increase in fluorescence with the primer/probe set Mal3 (grey) compared to Mal2 (brown), both of which are targeted against a *M. malmoense*-specific genomic sequence. ΔRn = normalised reporter value *i.e.*, change in fluorescent signal.

For *M. lepraemurium*, the primer/probe sets Lep1, Lep2, Lep3 and Lep4 were trialled on the provided gDNA sample extracted from one FFPE tissue biopsy sample. Testing with Lep3 showed a slight increase in the ΔRn value with this sample (Figure 7.2), but this curve also only consisted of the exponential-log phase. Testing with these four primer/probe sets on the remaining control samples did not show any positive responses.

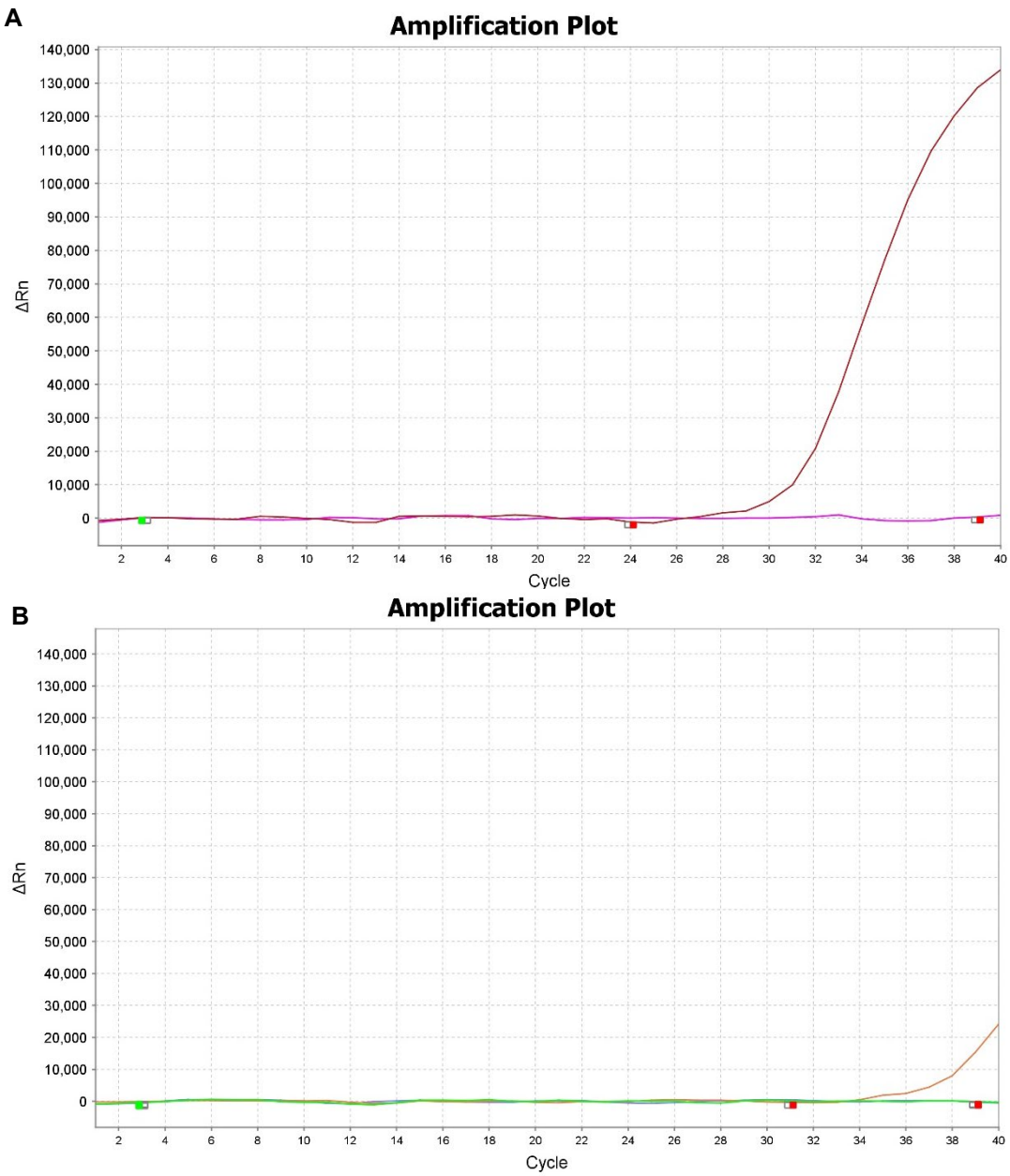


**Figure 7.2:** Amplification plot for qPCR on DNA previously extracted from a FFPE tissue biopsy sample from a cat with *M. lepraemurium* infection. There was detection of mycobacterial DNA with a positive response to the 16S rRNA marker (red), but testing with the Lep1 primer/probe was negative (pink) (A). There was an unconvincing increase in fluorescent signal using the Lep3 (orange) primer/probe, as the response only showed log-exponential growth rather than a complete curve. This primer/probe was targeted against the *M. lepraemurium* specific gene MLM_3300. There was no change in fluorescence with Lep2 (green) or Lep4 (lavender) (B).

## 7.3.2 Comparison of DNA Extraction Methods on Bovine and Feline FFPE Samples

Based on the results of primer/probe testing against MTBC gDNA extracted from culture material, the 16S rRNA, 18S rRNA, IS*6110* and RD-12 primer/probe sets were selected for testing on FFPE tissue biopsy samples from cases of natural or experimental MTBC infection. The water negative control results were used to set ΔRn values for $C_t$ determination for all three samples tested with each of the four extraction methods (Table 7.5). For 16S rRNA and 18S rRNA, a threshold of 40,000 was selected whereas for IS*6110* and RD-12 the threshold chosen was 20,000.

| **Sample/Method** | **DNA (ng/μL)** | **16S rRNA** | **IS*6110*** | **RD-12** | **18S rRNA** |
|---|---|---|---|---|---|
| *M. microti* – One | 854.90 | 29.7 | 27.0 | 30.9 | 23.7 |
| *M. microti* – Two | 426.07 | 30.3 | 27.4 | 30.6 | 23.3 |
| *M. microti* – Three | 4.12 | 36.8 | 31.9 | 35.0 | 27.3 |
| *M. microti* – Four | 886.76 | - | 30.7 | 35.1 | 39.9 |
| *M. bovis* – One | 77.74 | 34.8 | 38.5 | - | 24.6 |
| *M. bovis* – Two | 102.68 | 36.8 | 39.8 | - | 25.9 |
| *M. bovis* – Three | 3.12 | - | - | - | 29.7 |
| *M. bovis* – Four | 268.69 | - | - | - | 25.0 |
| *M. bovis* (bovine) – One | 694.45 | 36.5 | - | - | 26.3 |
| *M. bovis* (bovine) – Two | 528.48 | 38.1 | 37.4 | - | 27.6 |
| *M. bovis* (bovine) – Three | 58.79 | 37.6 | 38.6 | - | 25.6 |
| *M. bovis* (bovine) – Four | 372.59 | - | - | - | 28.6 |

**Table 7.5:** Comparison of $C_t$ values for four primer/probe sets across three FFPE tissue samples, using four different methods of DNA extraction. The greatest number of false-negative results, highlighted in red and boldface type, were reported using method four. No false-negative results were reported with method two. Across all three samples and four qPCR targets, $C_t$ values were similar between method one and method two. The concentration of extracted DNA from each sample was lowest with method three, while the concentration of DNA extracted from each sample with the remaining methods varied. There was no clear relationship between the concentration of extracted DNA and successful amplification of mycobacterial DNA.

Method four showed the greatest number of false negative responses, with mycobacterial DNA not identified in the *M. bovis*-infected feline or bovine FFPE tissue biopsy samples, as well as a negative 16S rRNA result for the feline *M. microti* sample, despite a positive result for both IS*6110* and RD-12. Method three gave a false negative result for 16S rRNA and IS*6110* in the feline *M. bovis* sample, whereas a false negative response was recorded for IS*6110* in the bovine *M. bovis* sample using method one; no false negative results were seen in samples extracted using method two. Across the four extraction methodologies $C_t$ values for each target showed variability within samples *e.g.*, RD-12 had similar $C_t$ values in the feline *M. microti* sample using methods one and two (30.9 and 30.6,

respectively) whereas the $C_t$ value was higher using methods three and four (35.0 and 35.1). In contrast, for 18S rRNA the lowest $C_t$ value in the bovine *M. bovis* sample was seen for method three. Overall, $C_t$ values were mostly lower for methods one and two but given the reduced processing time required for samples method one was selected for further development of the qPCR assay.

### 7.3.3 Further Validation of *M. lepraemurium*-specific Primer/Probe Sets

Having selected method one for DNA extraction from FFPE tissue biopsies, six samples from four cats with PCR-confirmed *M. lepraemurium* infection were identified from tissue archives and DNA was extracted and quantified. Two further *M. lepraemurium*-specific primer/probe sets were designed (Lep5 and Lep6) and tested, in addition to the previous four *M. lepraemurium*-specific primer/probe sets, 16S rRNA and 18S rRNA.

Of the six *M. lepraemurium*-infected FFPE tissue biopsies, five were positive on 16S rRNA *i.e.*, the peak ΔRn value exceeded that of the negative control, although for one of these samples the $C_t$ value was 38.2; the 16S rRNA negative sample came from the same cat as this sample with the high $C_t$ value, suggesting low levels of mycobacterial DNA were present in the tissue. All six samples were positive for 18S rRNA indicating adequate extraction of feline gDNA. For all six *M. lepraemurium*-specific primer/probe sets, a ΔRn threshold of 20,000 was set for determining sample positivity. Using this cut-off value, no samples were positive to Lep1, Lep5, or Lep6, but positive responses were reported to Lep2, Lep3, and Lep4. Lep3 showed the most promise, with three of six FFPE biopsies representing three of the four infected cats testing positive ($C_t$ values 27.7, 31.0 and 31.2, respectively); this included the FFPE biopsy sample that previously showed a very weak positive response to Lep3 (refer to Figure 7.2). This sample was also positive to Lep2 ($C_t$ = 34.3) and Lep4 ($C_t$ = 34.9) (Figure 7.3), having been previously negative for both primer/probe sets. This was the only sample that was positive to Lep4. The two other samples that were positive to Lep3 were also positive to Lep2, but with higher $C_t$ values (36.6 and 37.8, respectively).

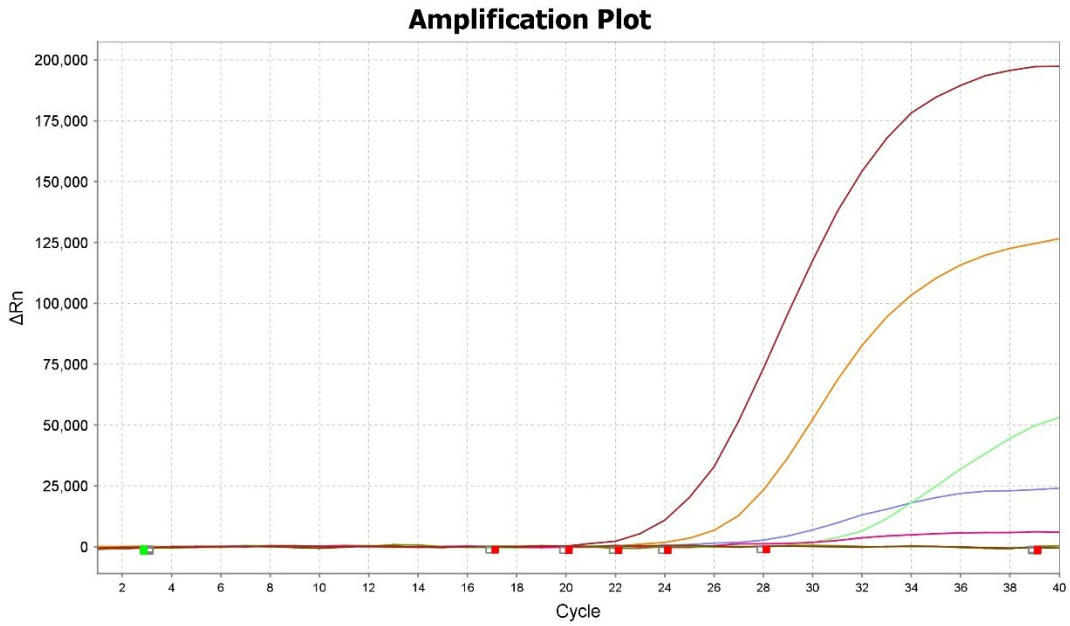


**Figure 7.3:** Repeated qPCR with the pan-mycobacterial 16S rRNA marker (red curve) and six *M. lepraemurium*-specific primer/probe sets from freshly extracted DNA following method one (see 7.2.3) from the same FFPE tissue block as in Figure 7.1. There was a strong positive response to the Lep3 marker (orange); less strong positive responses were identified with Lep2 (green) and Lep4 (lavender).

## 7.3.4 Final qPCR Primer/Probe List

Based on the results shown in 7.3.1 and 7.3.3, a final eight panel qPCR assay was decided upon (Table 7.6). The 16S rRNA and 18S rRNA pan-mycobacteria and mammalian DNA markers were included as endogenous controls. Members of the MTBC were identified using the IS*6110* primer/probe set (Halse *et al.*, 2010) as this gave better $C_t$ values than Ag85B. To differentiate between members of the MTBC of importance for feline mycobacterial infections, RD-1 TB and RD-12 (Halse *et al.*, 2011) were included; while RD-12 was positive to gDNA from *M. tuberculosis* and *M. microti* only, as predicted, none of the RD1 primer/probe sets showed an appropriate binding response as all were positive to *M. microti* and negative for *M. bovis*. However, RD-1 TB was selected to assess its performance against field strains of *M. bovis* present in feline mycobacterial FFPE biopsy samples. Novel primer/probe sets were included to detect the presence of *M. avium* (IS*1311*), *M. lepraemurium* (Lep3), and *M. malmoense* (Mal3).

| Name | Target | Complex/Species | Source |
|---|---|---|---|
| 16S rRNA | 16S rRNA | Pan-mycobacteria | This study |
| IS*6110* | IS*6110* | MTBC | Halse *et al*., 2010 |
| RD-1 TB | RD1 (Rv3875, *esxA*) | *M. bovis*<br>*M. tuberculosis* | Halse *et al*., 2011 |
| RD-12 | RD12 (Rv3120) | *M. microti*<br>*M. tuberculosis* | Halse *et al*., 2011 |
| IS*1311* | IS*1311* | *M. avium* | This study |
| Lep3 | MLM_3300 | *M. lepraemurium* | This study |
| Mal3 | MA2/6 | *M. malmoense* | This study |
| 18S rRNA | 18S rRNA | Mammalian DNA | This study |

**Table 7.6:** Final selection of primer/probe sets for qPCR on FFPE samples from cases of feline mycobacteriosis.

## 7.3.5 Results of qPCR on Feline Mycobacterial FFPE Tissue Biopsy Samples

A total of 38 FFPE tissue samples from cats with culture- or PCR-confirmed diagnosis of mycobacteriosis underwent DNA extraction and testing by qPCR using the final panel of primer/probes. Positive controls of gDNA from *M. tuberculosis*, *M. bovis*, *M. microti*, MAP, and *M. smegmatis* (n = 1 for each) and a negative control of PCR grade water (n = 1) were included and used to set ΔRn thresholds for $C_t$ calculations on FFPE test samples.

All five positive controls showed a strong positive response to the 16S rRNA primer/probe; from the negative control well a ΔRn threshold of 75,000 was set, giving $C_t$ values ranging from 21.6 to 32.0 for the positive controls. For IS*6110*, a threshold of 40,000 gave a $C_t$ value of 20.1 for *M. tuberculosis*, 27.3 for *M. bovis*, and 27.4 for *M. microti*, while MAP and *M. smegmatis* were IS*6110* negative, as expected. For the two RD markers, a threshold of 20,000 was set; for RD-1 TB a positive response was recorded for *M. tuberculosis* ($C_t$ = 26.3), but *M. bovis* was negative for this marker while *M. microti* was positive ($C_t$ = 33.7). For RD-12, positive responses were seen in both *M. tuberculosis* ($C_t$ = 23.0) and *M. microti* ($C_t$ = 29.4) and *M. bovis* was negative (Figure 7.4). The IS*1311* primer/probe was specific to MAP; a threshold of 25,000 gave a $C_t$ value of 26.6. Thresholds of 20,000 for the Lep3 and Mal3 primers were established from previously tested samples (see *7.3.1 Results of qPCR on Control gDNA Samples* and *7.3.3 Further Validation of* M. lepraemurium*-specific Primer/Probe Sets*); none of the MTBC nor NTM gDNA samples were positive for either marker. To indicate that feline DNA was present in the sample, an 18S rRNA threshold value of 120,000 was selected.

Twenty-five of the 38 FFPE tissue biopsies from cats with MTBC infections (*M. bovis* n = 10, *M. microti* n = 9, unclassified MTBC n = 6) underwent DNA extraction; the recovered concentration of DNA ranged from 3.11 – 2188.51ng/μL (median: 81.20ng/μL, mean: 281.13ng/μL). All samples were 18S rRNA positive, confirming the presence of mammalian DNA. A positive result was recorded in 13/25 (52%) samples for 16S rRNA, while 16/25 (64%) were IS*6110* positive; six samples that were IS*6110* positive ($C_t$ range 33.3 – 38.4) were 16S rRNA negative. Samples that were IS*6110* positive were interpreted as infected with MTBC. Three samples that were 16S rRNA positive ($C_t$ values 37.2, 37.8 and 40.0) were IS*6110* negative, indicating the presence of mycobacteria but not suggestive of MTBC infection. There was no positive response to any of the remaining mycobacterial primer/probe sets. Six samples were both 16S rRNA and IS*6110* negative; these six samples were also negative to all other primer/probe sets other than 18S rRNA.

Six of the 10 samples from cats with confirmed *M. bovis* infection were positive to IS*6110* (Cases 1, 3, 4, 7, 9, 10). Of these six, three were positive to RD-1 TB (Cases 1, 3, 9); the four samples that were IS*6110* negative were also RD-1 TB negative (Cases 2, 5, 6, 8). No positive responses to RD-12 were recorded, therefore the pattern of MTBC marker positivity indicated a diagnosis of *M. bovis* on qPCR in three out of 10 cats (Figure 7.5). Negativity to both RD-1 TB and RD-12 despite IS*6110* positivity was seen in three cats (Cases 4, 7, 10). This pattern of results confirms MTBC infection, and the lack of response to both RD markers could indicate infection with *M. bovis* BCG, although it is more likely these RD-1 TB negative responses result from a lack of sensitivity of the primer/probe set. No positive responses to the NTM markers (IS*1311*, Lep3, or Mal3) was reported for any of the IS*6110*-positive cats. One cat was 16S rRNA positive, but negative to all other mycobacterial primer/probe sets so a diagnosis of ‘other NTM infection’ was reached (Case 8), while the remaining three samples were negative to all mycobacterial primer/probes, so were considered ‘negative’ for mycobacterial infection (Cases 2, 5, 6).

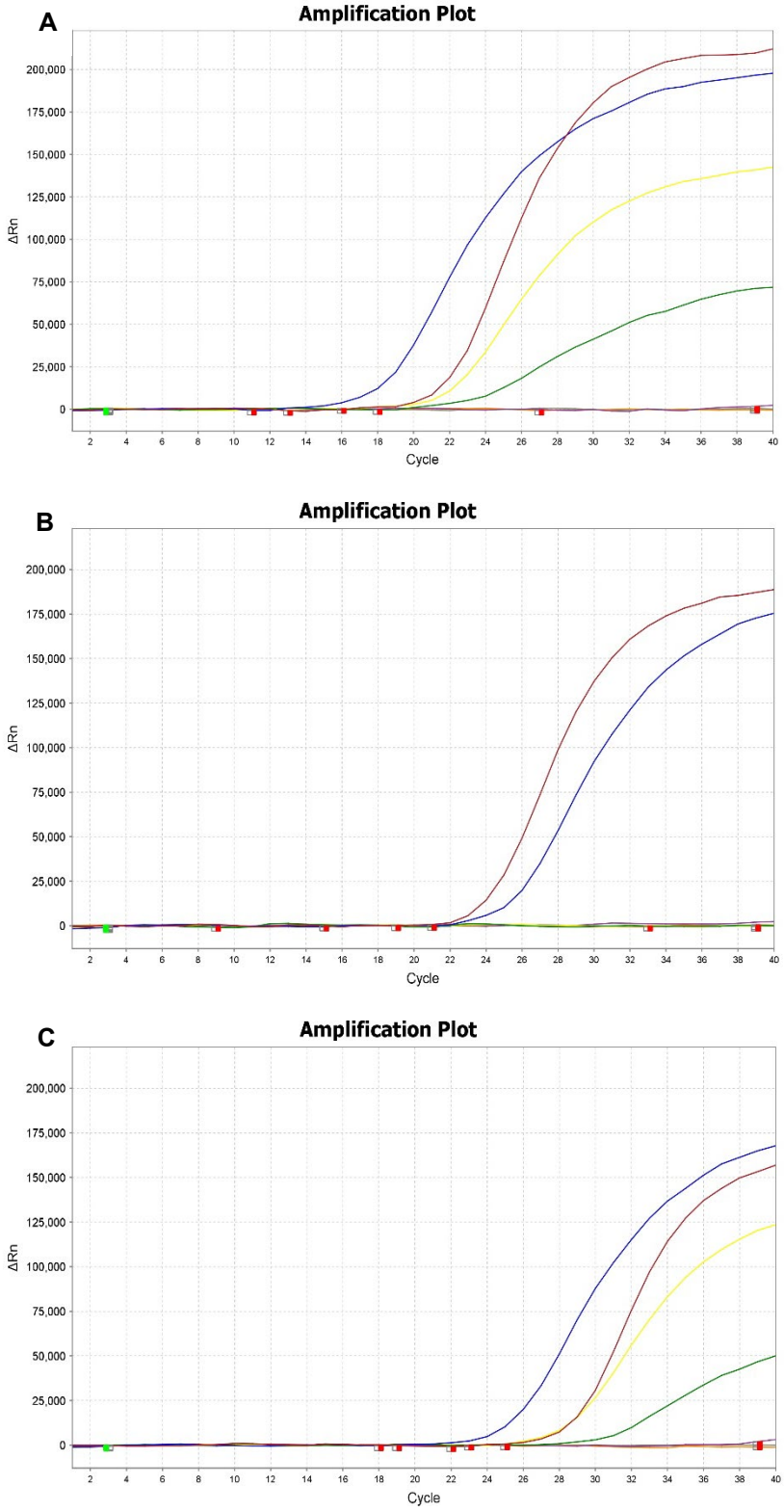


**Figure 7.4:** Results of qPCR testing on control gDNA from *M. tuberculosis* (A), *M. bovis* (B), and *M. microti* (C). All three samples were positive for the pan-mycobacterial 16S rRNA (red) marker, and MTBC-specific IS*6110* (blue). *Mycobacterium tuberculosis* showed the correct pattern of positivity to both RD-1 TB (green) and RD-12 (yellow) (A). *Mycobacterium bovis* was RD-12 negative as appropriate but was not positive to RD-1 TB *i.e.*, a false negative result. This pattern of results would indicate *M. bovis* BCG DNA (B). *Mycobacterium microti* was RD-12 positive but also showed positivity to the RD-1 TB primer/probe (C).

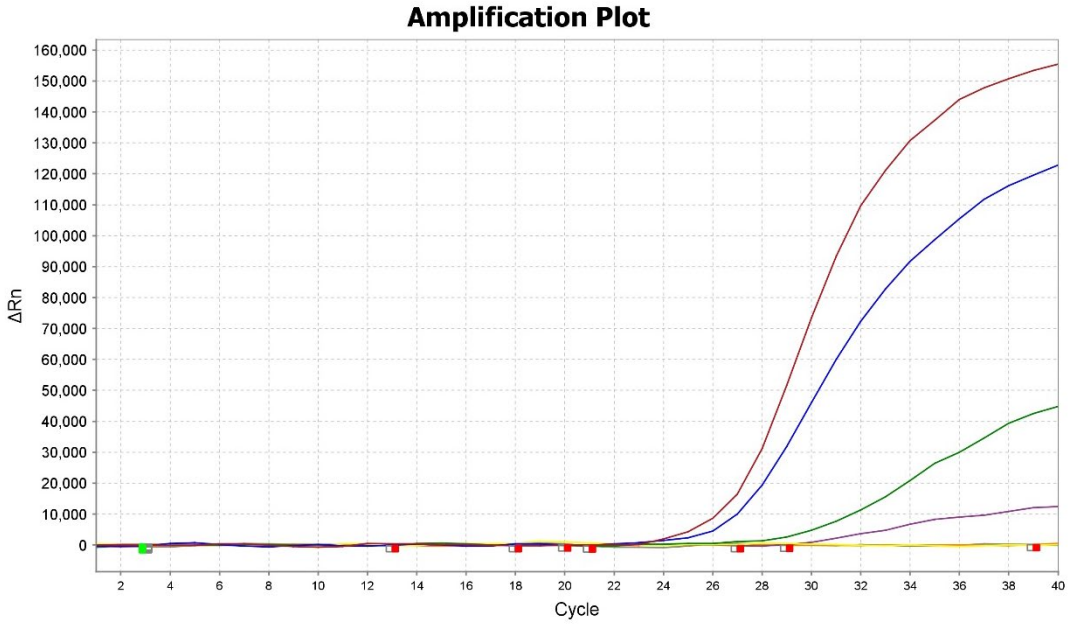


**Figure 7.5:** Results of qPCR on DNA extracted from a FFPE tissue biopsy sample from a cat with a PCR-diagnosis of *M. bovis* infection (Case 1). Unlike the control *M. bovis* sample (Figure 7.4 B) there was a positive response to the RD-1 TB primer/probe (green). This result, in combination with IS*6110* positivity (blue) and RD-12 negativity (yellow) is suggestive of *M. bovis*, hence there is agreement between qPCR and the official diagnosis. There is a small increase in the ΔRn value to the *M. avium*-specific IS*1311* marker, but this does not approach the cut-off threshold and may reflect non-specific amplification which could result from DNA damage following formalin-fixation.

Among the samples from nine cats infected with *M. microti*, six (Cases 11, 13-15, 17, 18) were positive to both IS*6110* and RD-12 while negative to RD-1 TB; this pattern of results was consistent with a diagnosis of *M. microti* (Figure 7.6). One cat (Case 16) was both 16S rRNA and IS*6110* positive, but negative to RD-1 TB and RD-12 which would suggest infection with *M. bovis* BCG. One cat was 16S rRNA positive only (Case 12), indicating 'other NTM infection', while the remaining cat (Case 19) was negative to all primer/probe sets. No response to IS*1311*, Lep3 or Mal3 were identified in any of these nine samples.

In cases that had been diagnosed with an MTBC infection, but species-level identification was not achieved, qPCR was 16S rRNA and IS*6110* positive in two cats, IS*6110* positive only in one cat, 16S rRNA positive only in another cat, and both 16S rRNA and IS*6110* negative in the final two cats. Therefore, a diagnosis of MTBC infection was made in three cats *i.e.*, those that were IS*6110* positive. One of these cats, which came from Highland, Scotland, was positive for RD-12, and RD-1 TB negative suggesting infection with *M. microti* (Figure 7.7). The cat that was 16S rRNA positive ($C_t$ = 40.0) did not show a positive response to any of the RD or NTM primer/probes, giving a diagnosis of 'other NTM infection', while the two 16S rRNA and IS*6110* negative samples were also negative for the remaining mycobacterial primer/probe sets, suggesting they were negative for mycobacterial infection on qPCR.

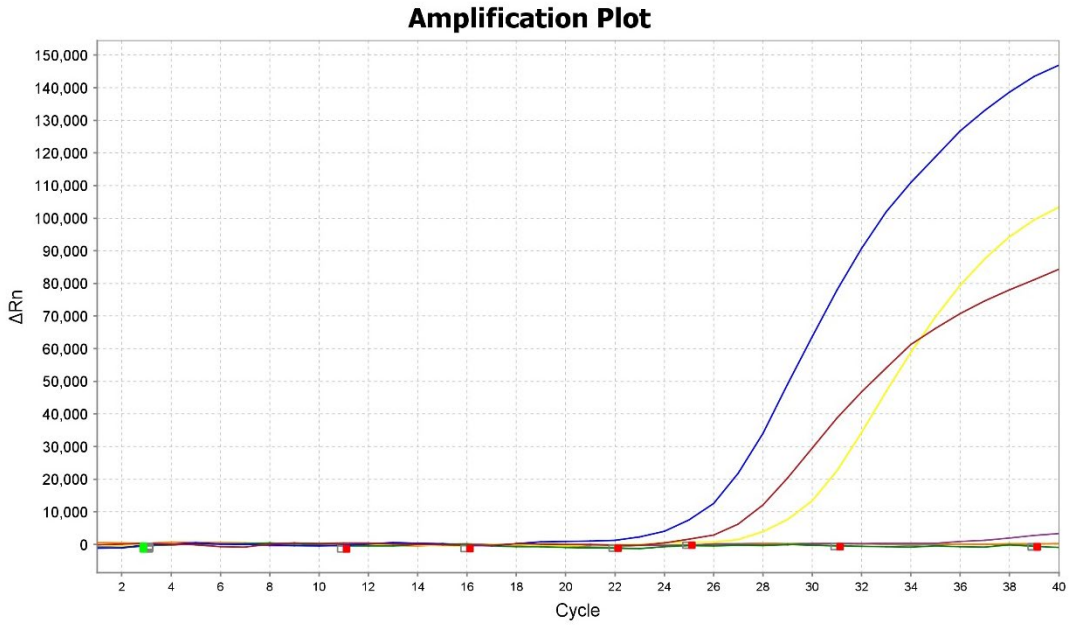


**Figure 7.6:** Results of qPCR on DNA extracted from a FFPE tissue sample from a cat with *M. microti* infection (Case 11). There was a positive response to the 16S rRNA (red), IS*6110* (blue), and RD-12 (yellow) markers, a pattern consistent with *M. microti* infection. Unlike the control gDNA sample, there was no positive response to RD-1 TB (green) (Figure 7.4 C).

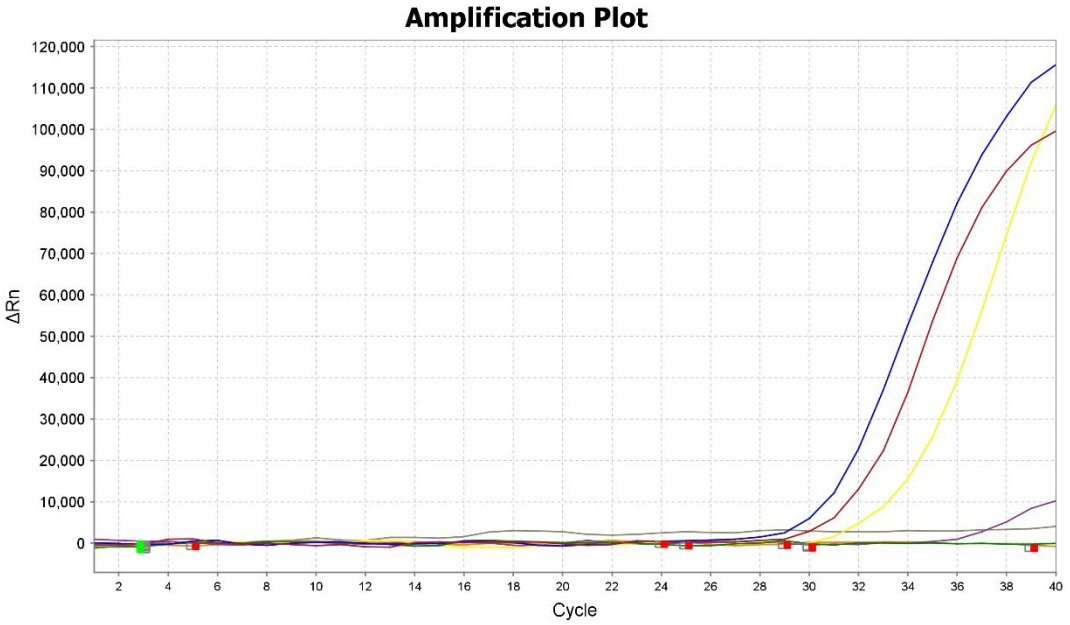


**Figure 7.7:** qPCR results on DNA extracted from a FFPE tissue sample from a cat from Highland, Scotland, that was previously diagnosed by PCR performed at Leeds Teaching Hospital NHS Trust with MTBC infection (Case 25); insufficient DNA was extracted and amplified for determination of which species of the MTBC was present. On qPCR the pattern of results suggests infection with *M. microti*: positive to 16S rRNA (red), IS*6110* (blue) and RD-12 (yellow), but negative for RD-1 TB (green).

The concentration of extracted DNA in the samples from the FFPE tissue biopsies of six cats with *M. avium* infection ranged from 31.76 – 1372.50ng/µL (median: 269.26ng/µL, mean: 412.68ng/µL). All six samples were 16S rRNA positive ($C_t$ range: 24.9-34.2), but only four were positive for the *M. avium*-specific marker IS*1311* ($C_t$ range: 26.1-38.6). These four samples were negative for the MTBC (IS*6110*, RD-1 TB, and RD-12), Lep3, and Mal3 primer/probe sets, therefore a diagnosis of *M. avium* was attributed to these samples on qPCR (Figure 7.8 A). The two samples that were IS*1311* negative were positive to Lep3 ($C_t$ 31.6 and 32.9, respectively), with no positive results to the MTBC or Mal3 primer/probes (Figure 7.8 B). Thus, they were diagnosed on qPCR with *M. lepraemurium* infection. Retrospective assessment of the case histories for these two cats showed that they were diagnosed with '*M. avium*-complex' infection on PCR by Leeds Teaching Hospitals NHS Trust.

There were four FFPE samples from cats with *M. lepraemurium* infection; the concentration of DNA ranged from 10.23 – 868.38ng/µL (median: 110.36ng/µL, mean: 274.83ng/µL). All four samples were 16S rRNA positive ($C_t$ range: 28.5-38.5), but only three were positive for the Lep3 marker only and were diagnosed on qPCR as being infected with *M. lepraemurium*. The remaining sample that was 16S rRNA positive but Lep3 negative was also negative to the other mycobacteria primer/probe sets, and was thus diagnosed with 'other NTM infection'.

Three FFPE samples from cats with other NTM infections were also selected for DNA extraction and testing with qPCR. One sample was from a cat with a culture-confirmed diagnosis of *M. malmoense*, but this was negative to all mycobacterial primer/probe sets including 16S rRNA and Mal3; this FFPE tissue biopsy was from a different cat than the sample that was used for validating the Mal3 primer/probe (see *7.3.1 Results of qPCR on Control gDNA Samples*). No positive responses on qPCR were reported for the sample from the cat diagnosed with *M. interjectum* infection by culture. Finally, the extracted DNA sample from a FFPE tissue biopsy of a cat with culture-confirmed *M. kumamotonense* infection was positive to both IS*6110* ($C_t$ = 36.4) and RD-12 ($C_t$ = 36.9), suggesting *M. microti*. A summary of results for all 38 cats is presented in Table 7.7.

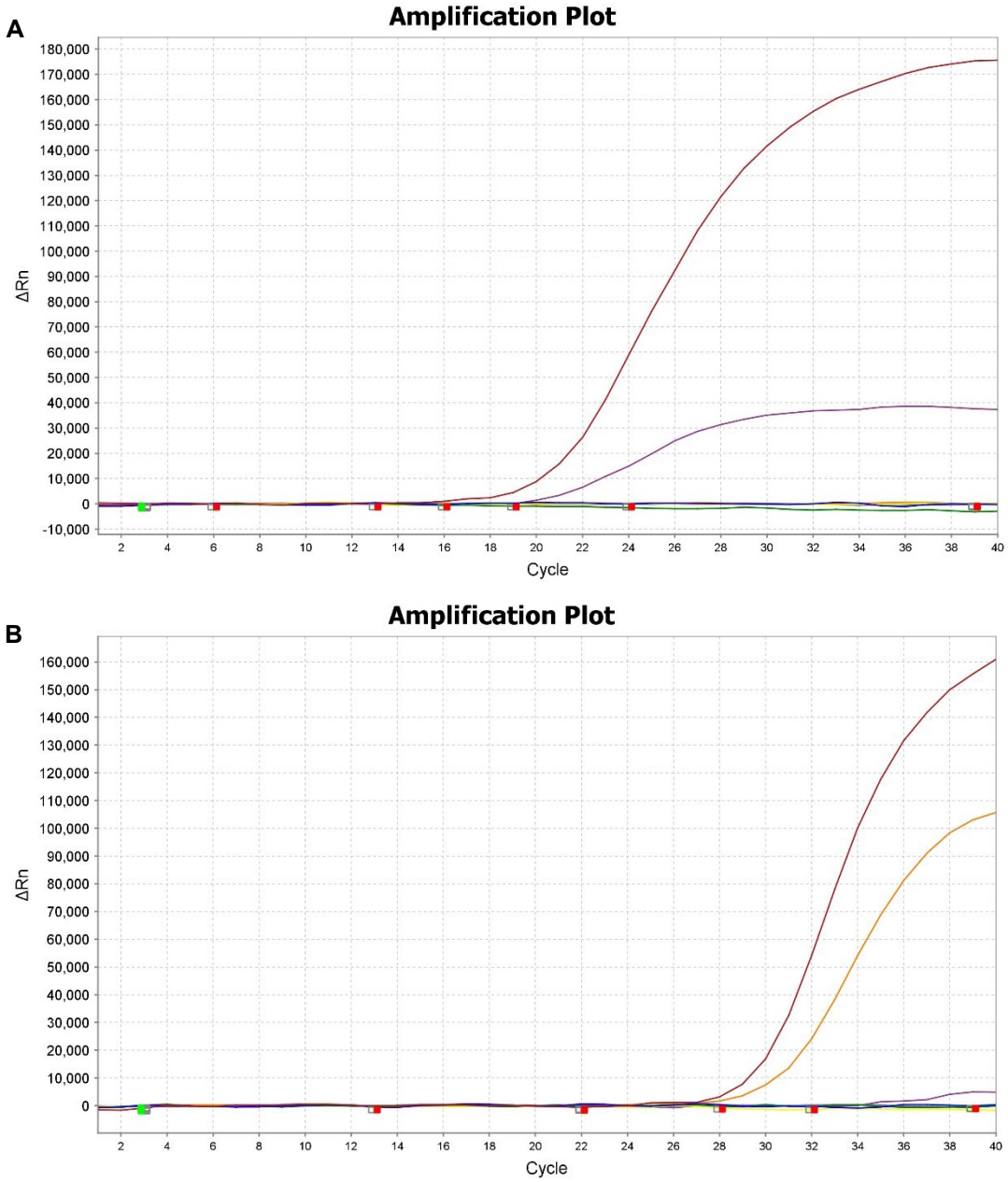


**Figure 7.8:** Results of qPCR testing on DNA extracted from FFPE tissue samples from two cats that had been previously diagnosed with *M. avium* infection by PCR. The qPCR diagnosis was consistent with *M.* avium infection (Case 27): positive to 16S rRNA (red) and IS*1311* (purple) (A). Two samples showed a pattern indicating infection with *M. lepraemurium*, with positivity to 16S rRNA and Lep3 (orange), and negative for IS*1311* (Case 28 shown here) (B).

| Case | Initial Diagnosis | Method | DNA (ng/µL) | 16S rRNA | IS*6110* | RD-1 TB | RD-12 | IS*1311* | Lep3 | Mal3 | qPCR Diagnosis |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *M. bovis* | PCR | 216.00 | 30.1 | 29.6 | 33.8 | - | - | - | - | *M. bovis* |
| 2 | *M. bovis* | Culture | 2188.51 | - | - | - | - | - | - | - | Negative |
| 3 | *M. bovis* | PCR | 774.78 | 38.7 | 35.2 | 38.9 | - | - | - | - | *M. bovis* |
| 4 | *M. bovis* | Culture | 238.76 | - | 36.4 | - | - | - | - | - | *M. bovis* BCG |
| 5 | *M. bovis* | Culture | 53.73 | - | - | - | - | - | - | - | Negative |
| 6 | *M. bovis* | Culture | 48.05 | - | - | - | - | - | - | - | Negative |
| 7 | *M. bovis* | Culture | 39.97 | - | 36.3 | - | - | - | - | - | *M. bovis* BCG |
| 8 | *M. bovis* | Culture | 18.64 | 37.8 | - | - | - | - | - | - | Other NTM |
| 9 | *M. bovis* | Culture | 14.21 | 36.4 | 36.6 | 39.2 | - | - | - | - | *M. bovis* |
| 10 | *M. bovis* | Culture | 34.44 | 36.8 | 38.4 | - | - | - | - | - | *M. bovis* BCG |
| 11 | *M. microti* | Culture | 854.90 | 37.1 | 28.4 | - | 30.7 | - | - | - | *M. microti* |
| 12 | *M. microti* | Culture | 242.10 | - | - | - | - | - | - | - | Negative |
| 13 | *M. microti* | Culture | 1051.30 | - | 35.1 | - | 35.9 | - | - | - | *M. microti* |
| 14 | *M. microti* | Culture | 217.02 | - | 35.6 | - | 38.7 | - | - | - | *M. microti* |
| 15 | *M. microti* | Culture | 140.20 | 39.6 | 35.4 | - | 38.8 | - | - | - | *M. microti* |
| 16 | *M. microti* | Culture | 114.06 | 39.8 | 38.3 | - | - | - | - | - | *M. bovis* BCG |
| 17 | *M. microti* | PCR | 158.49 | - | 33.3 | - | 35.8 | - | - | - | *M. microti* |
| 18 | *M. microti* | Culture | 30.62 | 38.7 | 38.9 | - | 38.7 | - | - | - | *M. microti* |
| 19 | *M. microti* | Culture | 72.28 | 37.2 | - | - | - | - | - | - | Other NTM |
| 20 | MTBC | PCR | 41.39 | - | - | - | - | - | - | - | Negative |
| 21 | MTBC | PCR | 8.50 | - | - | - | - | - | - | - | Negative |
| 22 | MTBC | PCR | 3.11 | 37.5 | 39.8 | - | - | - | - | - | *M. bovis* BCG |
| 23 | MTBC | PCR | 53.40 | - | 38.4 | - | - | - | - | - | *M. bovis* BCG |
| 24 | MTBC | PCR | 81.20 | 40.0 | - | - | - | - | - | - | Other NTM |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 25 | MTBC | PCR | 332.50 | 36.5 | 33.2 | - | 34.5 | - | - | - | *M. microti* |
| 26 | *M. avium* | PCR | 449.60 | 28.4 | - | - | - | 28.3 | - | - | *M. avium* |
| 27 | *M. avium* | PCR | 1372.50 | 24.9 | - | - | - | 26.1 | - | - | *M. avium* |
| 28 | *M. avium* | PCR | 88.91 | 34.2 | - | - | - | - | 32.9 | - | *M. lepraemurium* |
| 29 | *M. avium* | PCR | 486.70 | 25.7 | - | - | - | 38.6 | - | - | *M. avium* |
| 30 | *M. avium* | PCR | 46.60 | 29.4 | - | - | - | 30.7 | - | - | *M. avium* |
| 31 | *M. avium* | PCR | 31.76 | 32.9 | - | - | - | - | 31.6 | - | *M. lepraemurium* |
| 32 | *M. lepraemurium* | PCR | 868.38 | 28.5 | - | - | - | - | 28.2 | - | *M. lepraemurium* |
| 33 | *M. lepraemurium* | PCR | 65.02 | 32.3 | - | - | - | - | 31.1 | - | *M. lepraemurium* |
| 34 | *M. lepraemurium* | PCR | 10.23 | 38.5 | - | - | - | - | - | - | Other NTM |
| 35 | *M. lepraemurium* | PCR | 155.69 | 31.8 | - | - | - | - | 31.3 | - | *M. lepraemurium* |
| 36 | *M. malmoense* | Culture | 20.63 | - | - | - | - | - | - | - | Negative |
| 37 | *M. interjectum* | Culture | 28.76 | - | - | - | - | - | - | - | Negative |
| 38 | *M. kumamotonense* | Culture | 1123.68 | - | 36.4 | - | 36.9 | - | - | - | *M. microti* |

**Table 7.7:** Summarised results of qPCR testing on FFPE tissue biopsy samples from cats with culture or PCR-diagnosed mycobacterial infection, with $C_t$ values for each mycobacterial primer/probe set reported. Results that were considered false negative based on the culture or PCR diagnosis are highlighted in red and boldface type, while unexpected positive results are highlighted in blue. For the unclassified MTBC infections (cases 20-25), results of RD-1 TB and RD-12 could not be considered as false negative or unexpected positive results as the species-level diagnosis had not been achieved on initial diagnostic investigation. For all MTBC infections that were IS6110 positive, the pattern of positivity to RD markers were as follows: RD-1 TB positive/RD-12 positive – *M. tuberculosis*, RD-1 TB positive/RD-12 negative – *M. bovis*, RD-1 TB negative/RD-12 positive – *M. microti*, RD-1 TB negative/RD-12 negative – *M. bovis* BCG.

## 7.4 Discussion

This chapter presents data on the development of a novel qPCR assay for the diagnosis of feline mycobacterial infections from FFPE tissue samples, comparing methods to extract mycobacterial DNA from these samples and testing both published and novel primer/probe sets for a range of targets to allow for the complex and species-level identification of mycobacterial pathogens of importance for cats in the UK. Mycobacterial DNA was detected in 30/38 (78.9%) samples, but the results of qPCR did not always correlate with the culture- or PCR-confirmed diagnosis. Sixteen out of 25 (64%) samples with a culture or PCR diagnosis of MTBC infection were IS*6110* positive, confirming MTBC infection. For cases of *M. microti* infection, six out of nine were diagnosed as such on qPCR, while only three out of 10 cases of *M. bovis* infection gave a corresponding qPCR diagnosis. Infection with *M. avium* was suggested by qPCR in four out of six cases that had been diagnosed with *M. avium* on PCR; the two cases that were negative for the *M. avium*-specific marker IS*1311* were positive to a novel *M. lepraemurium* primer/probe set targeting the species-specific MLM_3300 gene. Three out of four cases of *M. lepraemurium* infection were positive to this marker, suggesting this may be an attractive marker for the qPCR diagnosis of infection with this species of mycobacteria. Mycobacterial complex- and species-specific primer/probe sets were also tested against NTM species that should not be detected by these primer/probes to determine if cross-reactivity may occur; one case of culture-confirmed *M. kumamotonense* infection responded to both the IS*6110* and RD-12 markers, which would suggest infection with *M. microti*. Therefore, while this test appears promising for the detection of some mycobacterial pathogens, namely *M. microti*, *M. avium*, and *M. lepraemurium*, further work is required to optimise mycobacterial DNA extraction to maximise qPCR sensitivity, to validate primers for regions of the genome specific to *M. bovis*, and to determine if cross-reactivity may occur with both mycobacterial and non-mycobacterial organisms.

While FFPE tissue samples represent an invaluable resource for diagnostic and research purposes (Fairley *et al.*, 2012, Kokkat *et al.*, 2013), there are some limitations that need to be overcome to maximise their clinical utility. The process of formalin-fixation results in the formation of cross-linkages between DNA and proteins (Hoffman *et al.*, 2015), and while this cross-linking of macromolecules helps to preserve cellular and tissue integrity for histopathological analysis, it inhibits the extraction and amplification of DNA, while the use of unbuffered fixatives can result in fragmentation of DNA compromising the ability to detect longer target sequences (Gilbert *et al.*, 2007). There are also many other intrinsic factors regarding tissue fixation that can compromise the quality and quantity of extracted DNA such as the temperature of the fixative and the duration of fixation, in addition to pre- and post-fixation parameters including how the sample was obtained, and the storage conditions of the sample post-fixation (Greer *et al.*, 1991, Srinivasan *et al.*, 2002, Miething *et al.*, 2006, Gilbert *et al.*, 2007). The FFPE tissue blocks from which DNA was extracted in this study were submitted by the PVS, private diagnostic histopathology laboratories, or the VPU; therefore, there is an inherent lack of consistency by which the tissue samples were taken and fixed, which could compromise the ability to successfully

extract DNA. Having greater control of sample collection and storage could help minimise some of these variables and subsequently improve DNA extraction.

Methods of DNA extraction from FFPE tissues can prove challenging, but various techniques have been investigated and described to maximise recovery of nucleic acids and these appear to be successful for use on mammalian FFPE tissues (Díaz-Cano and Brady, 1997, Gilbert *et al.*, 2007, Lin *et al.*, 2009, Weiss *et al.*, 2011, Sengüven *et al.*, 2014). However, extracting DNA from mycobacteria adds further complications; the thick, waxy mycobacterial cell wall requires lysis with either toxic chemicals, enzymatic digestion, bead-beating, or the use of mycobacteriophages (Gonzalez-y-Merchand *et al.*, 1996, Catalão and Pimentel, 2018, Epperson and Strong, 2020). As such, there is no standardised approach to the extraction of mycobacterial DNA from FFPE tissue samples (Kipar *et al.*, 2003, Johansen *et al.*, 2004, Davies *et al.*, 2006, Fyfe *et al.*, 2008, Surat *et al.*, 2014), making selection of an appropriate methodology challenging. However, the use of FFPE tissues may be considered preferable by some because these samples do not require handling at Advisory Committee of Dangerous Pathogens (ACDP) Containment Level 3, unlike fresh tissue samples from cases of infection with *M. bovis*, *M. microti*, and *M. malmoense*, which are all Hazard Group 3 organisms (Health and Safety Executive, 2021). Therefore, optimising methods of DNA extraction from FFPE tissues from cats with mycobacterial infection is essential to fully unlock the diagnostic potential of these samples. This study investigated four methods of DNA extraction incorporating different approaches to paraffin extraction and tissue digestion conditions and methods. Methods one and two (Fyfe *et al.*, 2008, Reppas *et al.*, 2013, Surat *et al.*, 2014) were broadly similar regarding the quantity and quality of DNA that was extracted, as well as the $C_t$ values obtained for the different primer/probe sets trialled. Method three (O'Halloran, 2019) incorporated a bead-beating step and prolonged tissue digestion – a minimum of 48 hours – but the concentration of DNA obtained from samples was lower than methods one and two. Additionally, this method incorporated the use of SLS with lysozyme, but there is evidence to suggest that SLS can form a stable complex with lysozyme, inhibiting its activity (Imoto *et al.*, 1979). Despite this, mycobacterial DNA was successfully amplified from two of the three samples tested, although the $C_t$ values were higher for the *M. microti*-infected feline sample using this method compared to methods one and two. In commercial laboratories automated methods for the extraction of mycobacterial DNA from FFPE tissue samples are preferred as these reduce processing time and appear superior to manual DNA extraction (Caldarelli-Stefano *et al.*, 1999, Huijsmans *et al.*, 2010, Surat *et al.*, 2014). Such automated methods may be worthwhile investigating for the development of a commercial qPCR diagnostic assay for cases of feline mycobacteriosis.

Other factors that could influence the extraction of DNA and successful amplification by qPCR include the amount of starting material and slice thickness of FFPE tissue sections, the presence of PCR inhibitors, the composition of the qPCR master mix, and the conditions for qPCR. Studies on FFPE tissue samples for PCR testing in cases of feline mycobacteriosis have used variable numbers and thickness of sections, from as few as one 30-micron tissue slice (Kipar *et al.*, 2003) to at least six 10 to 20-micron sections (Fyfe *et al.*, 2008, Reppas *et al.*, 2013). Increasing the amount of starting material would increase the amount of mycobacterial DNA present within the sample, but high levels of

fragmented DNA can inhibit *Taq* polymerase activity (Dietrich *et al.*, 2013) while qPCR for bacterial DNA may be considered unreliable if there is excessive host DNA in the extract (Radomski *et al.*, 2013, Flores Bueso *et al.*, 2020). It has been suggested that increasing the concentration of polymerase and starting nucleotides, and lengthening cycling times may help overcome this inhibition by host DNA (Dietrich *et al.*, 2013). Excessive host DNA may explain why negative results for mycobacterial markers were recorded for some samples in this study with a high starting concentration of DNA, as Nanodrop quantification does not discriminate between mycobacterial or host DNA. Bacterial Index scoring of ZN-stained sections from FFPE tissue blocks prior to selection for DNA extraction and subsequent qPCR would allow for samples to be chosen with a high BI to maximise the amount of mycobacterial DNA present for extraction. Slice thickness may also play an important role, as it has been shown that sections greater than two microns thick and with a digestion time of less than 48 hours have a reduced yield of DNA (Weiss *et al.*, 2011). Thinner slices may be easier to digest, allowing for improved DNA extraction and reversal of cross-linkages. In addition to excessive levels of host DNA, other PCR inhibitors may be present in the samples. These include residual xylene if samples were insufficiently washed (Coura *et al.*, 2005), SLS and lysozyme (Rossen *et al.*, 1992), and haemoglobin (Schrader *et al.*, 2012). Some of these factors may be difficult to control for, such as haemoglobin in samples that contain numerous erythrocytes, but other inhibitors like xylene and SDS can be overcome by thorough washing, or use of alternative reagents; however, these would need to be investigated for their own capacity as an inhibitor of PCR.

Development of any new diagnostic assay requires validation against known positive and negative controls to establish cut-off thresholds and to ensure sensitivity and specificity of the test. In this study, mycobacterial primer/probe sets were tested against extracted gDNA from cultures of four MTBC pathogens (*M. tuberculosis* H37Rv, *M. bovis* AF2122/97, *M. bovis* BCG Pasteur 1173P2, and *M. microti* NCTC 8710), and two NTM organisms (MAP K10, and *M. smegmatis* NCTC 8519). Extracted DNA from one FFPE tissue sample from a cat with culture-confirmed *M. malmoense* infection, and from six FFPE biopsies from cats with PCR-diagnosed *M. lepraemurium* infection were also used for primer/probe validation to increase the scope of species-specific mycobacterial testing possible from previous attempts to develop a PCR-based diagnostic using FFPE tissues (O'Halloran, 2019). For a more robust diagnostic assay, extended validation against gDNA from more mycobacterial species, in addition to other genera of bacteria, should be performed to truly determine whether these primer/probe sets truly are mycobacterial-specific (Lorente-Leal *et al.*, 2019). For example, *Rhodococcus* spp. have been frequently identified in pyogranulomatous lesions from cats with no demonstrable acid-fast organisms, as well as in control tissues from cats and dogs with non-mycobacterial inflammatory or neoplastic cutaneous lesions and control tissues from other organs (liver, spleen, and lung) (Ramiro-Ibanez *et al.*, 2004). It has been suggested that the presence of *Rhodococcus* spp. in these tissues are due to contamination rather than these organisms being the cause of disease, especially if the number of mycobacterial organisms in a lesion is very low (Foley *et al.*, 2004, Ramiro-Ibanez *et al.*, 2004, Davies *et al.*, 2006). Having said that, *Rhodococcus* spp. have been infrequently identified as a cause of cutaneous disease in cats (Fairley and Fairley, 1999, Patel, 2002, Takai *et al.*, 2003, Farias *et al.*, 2007,

Aslam *et al.*, 2020) Given this organism is closely related to mycobacteria, *Rhodococcus* spp. may generate a false-positive result when using putatively mycobacterial-specific 16S rRNA primer/probes. This may erroneously identify the presence of mycobacteria in a sample where there is disease due to *Rhodococcus* spp., or simply contaminated with *Rhodococcus* spp. which may result in either unnecessary treatment, or treatment with an inappropriate protocol of antimicrobial drugs. In this study, all mycobacterial control samples were positive for the pan-mycobacterial marker 16S rRNA, while MAP K10 was the only control positive for the *M. avium*-specific marker IS*1311* (Shin *et al.*, 2010). Extracted DNA from the FFPE tissue block from the cat with *M. malmoense* infection was the only control positive for Mal3, a novel primer/probe set designed against the *M. malmoense*-specific MA2/6 sequence (Kauppinen *et al.*, 1999), and Lep3 was only detected in the control DNA samples from cats infected with *M. lepraemurium*.

Primer/probe sensitivity and specificity was less straightforward for the MTBC pathogens. All four MTBC control samples were positive for Ag85B and IS*6110* (Halse *et al.*, 2010), but only *M. tuberculosis* was positive for *mpb70* despite reported positivity for fresh tissue samples from cattle infected with *M. bovis* (Lorente-Leal *et al.*, 2019). As *mpb70* is present as a single copy gene in *M. bovis*, and its corresponding isoform *mpt70* in *M. tuberculosis* (Wiker, 2009), the amount of *mpb70* DNA in the negative MTBC samples may have been below the limit of detection of this assay. Quantification and standardisation of the DNA concentration for the control samples derived from cultured isolates would have allowed for assessment of the limit of detection for each primer/probe set across organisms. While both primer/probe sets against Ag85B and IS*6110* were specific to the MTBC when tested against control gDNA in this study, IS*6110* was chosen as the MTBC target because it is MTBC-specific, whereas Ag85B is present in NTM such as *M. avium*, *M. smegmatis*, *M. kansasii*, *M. intracellulare*, and *M. leprae* (Zhang *et al.*, 2018). Although the genetic sequence of Ag85B differs between MTBC and NTM species, allowing for the design of MTBC-specific primer/probe sets, IS*6110* is a well-recognised MTBC-specific marker (Aranaz *et al.*, 1996, Caldarelli-Stefano *et al.*, 1999, Lauren Steinlein *et al.*, 2002, Costa *et al.*, 2014, Surat *et al.*, 2014) and gave lower $C_t$ values for MTBC controls than Ag85B.

Species-level identification within the MTBC can be accomplished by targeting the RD portions of the genome (Faksri *et al.*, 2016). These are regions which vary in their presence across the members of the MTBC, and the pattern of RD absence has been used to explain how different species of MTBC arose (Brosch *et al.*, 2002, Brites *et al.*, 2018). In the classic paradigm, *M. microti* is recognised as a naturally occurring RD1 mutant deletant (Brodin *et al.*, 2002, Pym *et al.*, 2002, Orgeur *et al.*, 2021), while *M. bovis* is RD1 positive, but has lost other parts of the genome compared to *M. microti* namely RD4 and RD12 (Faksri *et al.*, 2016). *Mycobacterium bovis* BCG is characterised by the absence of RD4 and RD12, as for *M. bovis*, but also RD1; however, the RD1 deletion in *M. bovis* BCG is not identical to that of *M. microti* (Brodin *et al.*, 2002). Subsequently, these deletions have been termed $RD1^{mic}$ and $RD1^{bcg}$. While there is some overlap, these deletions are not identical (Brodin *et al.*, 2002, Huard *et al.*, 2003). Both *M. bovis* and *M. microti* are negative for RD7, RD8 and RD9, while these are present in *M. tuberculosis* (Faksri *et al.*, 2016). In this study, the *M. tuberculosis* control was detected appropriately by all RD primer/probe sets. Similarly, the *M. bovis* BCG control was negative for all RD primer/probe

sets. The responses of *M. bovis* and *M. microti*, however, were not as expected. While the *M. bovis* control was negative to the primer/probe sets targeting RD4, RD9 and RD12, it was also unexpectedly negative for all five RD1 primer/probes. An extracted gDNA sample from the *M. bovis* AF2122/97 reference strain was used in this study (O'Halloran, 2019); given this was negative to all five RD1 primer/probes it would suggest that either there has been loss of these genes from repeated subculture of individual colonies as has been demonstrated for *Escherichia coli* and *S. enterica* (Knöppel *et al.*, 2018), or the sample had been mislabelled as *M. bovis* when in fact it was extracted gDNA from *M. bovis* BCG. The two novel RD1 primer/probe sets used in this study were designed from the amplified region of the *esxA* (Rv3875) gene that had been shown to be present in *M. bovis* using conventional PCR (O'Halloran, 2019), raising further questions regarding the lack of detection of RD1 in this study. In contrast, gDNA extracted from *M. microti* NCTC 8710 was positive to all five RD1 primer/probes, which are targeted against the genes *esxA* (Rv3875) and *esxI* (Rv3876). While Rv3876 is considered partially present in *M. microti*, and absent in *M. bovis* BCG, Rv3875 should be absent from both organisms (Brodin *et al.*, 2002). However, Rv3875 has been identified in some field strains of *M. microti*, indicating that RD1$^{mic}$ deletions can vary between strains (Frota *et al.*, 2004). Regarding performance of the other RD primer/probe sets, *M. microti* showed a positive response to the primer/probe targeting RD12, and was not detected by those targeting RD9, which is as expected based on currently available data on the genome structure of this pathogen (Frota *et al.*, 2004, Faksri *et al.*, 2016). For the two primer/probe sets designed against RD4, there was an increase in the fluorescent signal, but this was not sufficient to call the response as positive, hence this target was dropped for further exploration of test samples; additionally, its role was mirrored by that of the primer/probe against RD12 which showed better test performance on control gDNA. Despite the inappropriate responses to the RD1-targeted primer/probe sets, one of these was selected for further testing on clinical samples to determine whether positivity could be recorded in cases of *M. bovis* infection, and whether there would be a response in cases of infection with *M. microti*.

Mycobacterial DNA, indicated by positivity to any of the seven mycobacteria-specific primer/probe sets, was detected in nearly 80% of samples (30/38), suggesting that the DNA extraction technique was suitable, but could be further optimised, especially as a species-level diagnosis was not possible in all 30 samples from which mycobacterial DNA was detected. For the complex-level identification of MTBC DNA from FFPE tissue samples from cats with a culture- or PCR-confirmed diagnosis of *M. bovis*, *M. microti*, or an unclassified MTBC infection, 64% of samples (16/25) were IS*6110* positive. This target gene is often found in multiple copies in MTBC organisms *e.g.*, 16 copies are present in *M. tuberculosis* H37Rv (Antoine *et al.*, 2021), making it an attractive target for the identification of MTBC pathogens in FFPE tissue samples, but the number of IS*6110* copies can be much smaller. For example, some strains of *M. bovis* and *M. bovis* BCG may only have one or two copies of IS*6110* (Fomukong *et al.*, 1992, Alonso *et al.*, 2013), which may explain why only six out of 10 FFPE samples from cats with *M. bovis* were IS*6110* positive. A slightly higher number of samples coming from cats with *M. microti* infection were IS*6110* positive (seven out of nine); genomic analysis of *M. microti* isolates have

identified between eight and 15 copies of this gene in this organism (Orgeur *et al.*, 2021), which may suggest why it was slightly more sensitive compared to IS*6110* testing on *M. bovis* samples.

Compared to the control samples, testing for the RD markers appeared more appropriate on the FFPE tissue samples. The selected RD1 primer/probe set (termed RD-1 TB in this study) (Halse *et al.*, 2011), despite only giving a positive result in three out of the 10 *M. bovis* samples, was not positive for any of the nine *M. microti* samples. Meanwhile, the RD12 primer/probe (Halse *et al.*, 2011) was positive in six of the nine *M. microti* samples, with no positive results for any of the *M. bovis* samples. This would suggest reasonable performance of the RD12 primer/probe set, but there is poor discriminatory power of the RD1 primer/probe set tested. Testing of the other primer/probe sets designed against RD1 on the clinical FFPE *M. bovis*-infected samples could suggest whether the RD-1 TB primer/probe set simply performed poorly on these samples and would require optimisation of the qPCR protocol (Bustin and Huggett, 2017), or whether there are inherent characteristics of the primer/probe that result in poor efficiency in binding to template DNA (Svec *et al.*, 2015). Overall, there was agreement between qPCR and culture/PCR in three out of 10 cases of *M. bovis* infection, and six out of nine cases of infection with *M. microti*. For the six cases that had a PCR diagnosis of an unclassified MTBC infection, only one sample showed a response to either of the RD primer/probe sets. This was from a cat living in Scotland that was IS*6110* positive, RD12 positive, RD1 negative, which would suggest infection with *M. microti*. Given the distribution of cases of *M. bovis* and *M. microti* infections in cats across the UK (Smith *et al.*, 2009, Gunn-Moore *et al.*, 2011a), these epidemiological findings support the qPCR diagnosis of *M. microti* infection. While these results should not be over-interpreted given the poor sensitivity of the RD-1 TB primer/probe in *M. bovis* infected samples, it was encouraging that none of the unclassified MTBC infections tested positive to both RD1 and RD12, which would have suggested infection with *M. tuberculosis*.

For samples that were both RD1 and RD12 negative, but IS*6110* positive, a nominal diagnosis of *M. bovis* BCG was made on qPCR. A case of *M. bovis* BCG infection in a cat has been recorded (Manou *et al.*, 2021), but as seven out of 10 *M. bovis*-infected samples were negative to RD-1 TB a subsequent diagnosis of *M. bovis* BCG based on lack of detection of RD targets suggests that this qPCR likely over-diagnoses infection with this organism; a more appropriate diagnosis in such cases would be 'MTBC infection'. To actively identify *M. bovis* BCG, other targets should be investigated such as those that are specific to $RD1^{mic}$ and $RD1^{bcg}$ (Faksri *et al.*, 2016). In this scenario, *M. bovis* would be positive for both genes (as would *M. tuberculosis*), *M. microti* would be $RD1^{mic}$ negative and $RD1^{bcg}$ positive, whereas *M. bovis* BCG would be $RD1^{mic}$ positive and $RD1^{bcg}$ negative. Targeting RD9 would then allow for the discrimination between *M. bovis* and *M. tuberculosis*. Feline infections with *M. bovis* BCG could arise from shedding of viable organisms into the environment by immunocompromised badgers post-vaccination and subsequent secondary contamination of wounds as hypothesised in the singular case report of *M. bovis* BCG infection in a cat (Manou *et al.*, 2021). However, this would result in feline infections only being reported from specific regions where such trial vaccination schemes are taking place (Benton *et al.*, 2020). Also, while the BCG vaccine uses a live attenuated strain and there is potential reversion to virulence, cases of disease in humans due to BCG are rare and usually seen in

individuals who are immunocompromised (Cuello-Garcia *et al.*, 2013). Furthermore, rates of BCG vaccination in children in England have declined following policy changes introduced in 2005 (Nguipdop-Djomo *et al.*, 2014) and are now only offered to those considered at higher risk of TB, so this greatly reduces the chance of human-to-cat transmission of *M. bovis* BCG given the reduced number of children who now receive the vaccine. Altogether this would suggest the cases presented here with a pattern of qPCR results suggestive of *M. bovis* BCG infection more likely reflect poor sensitivity of RD primer/probe sets and potentially very low levels of mycobacterial DNA present in the tested samples.

Results of qPCR on DNA extracted from FFPE biopsy samples from cats with *M. avium* infection were promising; four out of six were positive using a novel primer/probe set targeted against the *M. avium*-specific marker IS*1311*. This marker has been used for the identification of *M. avium* subspecies (Shin *et al.*, 2010) and it is present in multiple copies, with seven IS*1311* copies identified in MAP K10 (Johansen *et al.*, 2005). This target has also been used, in conjunction with IS*1245*, for molecular epidemiological studies into *M. avium* species arising from different host species (Whittington *et al.*, 1998, Johansen *et al.*, 2007). However, positivity to IS*1311* has been shown in other NTM including *M. malmoense* (Keller *et al.*, 2002); no positive response to IS*1311* was seen in the control *M. malmoense* DNA extracted from a FFPE tissue sample, and there was no successful amplification of mycobacterial DNA extracted from a second *M. malmoense* FFPE sample, so this lack of specificity cannot be commented on for this study. Further validation of this primer/probe set on gDNA from cultured isolates of *M. malmoense* would be required to provide further assessment of species-specificity.

While four of the *M. avium*-infected FFPE tissue samples were positive for the IS*1311* marker, two were negative; instead, they showed a positive response to a novel primer/probe set (Lep3) against the gene MLM_3300 which is specific to *M. lepraemurium* (Benjak *et al.*, 2017). Three of the four cats that had a PCR diagnosis of *M. lepraemurium* were also Lep3 positive on qPCR, indicative of good test performance for this species of mycobacteria. While *M. lepraemurium* does not appear to be pathogenic to humans, it is important to recognise cases of infection in cats because they typically have an good prognosis (O'Brien *et al.*, 2017c, Ghielmetti *et al.*, 2021) and can be successfully treated without the requirement for drugs such as rifampicin (Malik *et al.*, 2002, O'Brien *et al.*, 2017c), which are typically associated with side effects in cats (O'Halloran and Gunn-Moore, 2017). Rifampicin should also be safeguarded as a key antimicrobial drug for human health (World Health Organization, 2019a). For the two cats that had a PCR diagnosis of *M. avium* but had a qPCR result suggestive of *M. lepraemurium* infection, retrospective assessment of the diagnostic report showed that they had a diagnosis of '*M. avium*-complex' infection. The term MAC can have multiple definitions; for some, it simply refers to the subspecies of *M. avium* (Shin *et al.*, 2010), others include *M. intracellulare* in this description (O'Halloran and Gunn-Moore, 2017), while others have suggested an expanded definition which includes *M. lepraemurium* (van Ingen *et al.*, 2018). However, it is important to differentiate between infection with *M. avium* or *M. lepraemurium*, as *M. avium* infections are notoriously more difficult to successfully treat (Munro *et al.*, 2021). At the time of diagnosis for these two cats, 16S rRNA sequencing may have generated a product that most closely mapped to *M. avium* based on sequence data available in online

databases, especially if sequence data was not available for *M. lepraemurium*. An alternative explanation is co-infection with both *M. avium* and *M. lepraemurium*, and these differing methodologies detected DNA to one organism, but not both. These two PCR *M. avium*/qPCR *M. lepraemurium* cats came from the Isle of Man; there is no published data on the epidemiology of feline mycobacterial infections from this location, but two of the PCR and qPCR *M. lepraemurium* positive samples also came from the Isle of Man, which may suggest a local burden of infection with this pathogen.

Testing on other NTM infections was less rewarding. Despite identification of marker that appeared to be specific for *M. malmoense* (Mal3) based on DNA that had been previously extracted from a FFPE tissue block from a cat infected with this organism (O'Halloran, 2019), there was a failure of detection of mycobacterial DNA from a second FFPE tissue sample from another cat infected with *M. malmoense*. This pathogen has been sporadically identified in cats in GB (Gunn-Moore *et al.*, 2011a) and it is an important cause of disease in humans (Doig *et al.*, 2002, Russell *et al.*, 2014), hence why it was included for specific detection in this qPCR. Primer/probe sets should be tested on gDNA from reference strains of this organism to confirm sensitivity, but of importance was that no positive responses were identified from samples containing DNA from other mycobacterial species, providing some information on the specificity of this novel primer/probe set. Samples from two cats infected with NTM that should not give a positive response to any of the mycobacterial primer/probes other than for 16S rRNA were also selected for tested to check for cross-reactivity. While there was no positive detection of mycobacterial DNA, including for 16S rRNA, for the sample from the cat with *M. interjectum* infection, there were positive results for sample from the *M. kumamotonense*-infected cat. However, it was not positive for 16S rRNA, but instead was positive for both IS*6110* and RD12; this pattern of results suggested *M. microti* infection. Misidentification of *M. kumamotonense* as a member of the MTBC has been reported in a human with cervical lymphadenitis (Rodríguez-Aranda *et al.*, 2010), but this was based on testing for the 16S rRNA gene rather than identification of IS*6110* and RD12 DNA. This pathogen was first reported in 2006 and appears to be most closely related to the *M. terrae*-complex (Masaki *et al.*, 2006), and it has been detected by molecular testing from granulomatous lesions in both a weasel and a stoat in the south-west of England (Simpson *et al.*, 2016). Like members of the MTBC, *M. kumamotonense* is a slow-growing pathogen (Masaki *et al.*, 2006), therefore it is not likely that the culture-confirmed diagnosis resulted from more rapid growth of this organism, outcompeting any MTBC pathogen that may have also been present, hence it was identified whereas *M. microti*, as suggested by the results of qPCR, was not isolated. However, the possibility of a mixed infection cannot be ruled out; the lesion that was sampled and submitted for culture may have been different to the lesion that was biopsied for histopathology, hence the discordant culture/qPCR result. Contamination of the sample during DNA extraction with *M. microti* DNA was considered unlikely, as all FFPE tissue blocks were cut with a fresh microtome blade, and this sample was processed at the same time as the *M. interjectum* and *M. bovis* samples rather than in conjunction with samples infected with *M. microti*. A final possibility is that *M. kumamotonense* was cultured as a contaminant organism; it has been identified in fish (Mrlik *et al.*, 2012), therefore water sources may act as an ecological niche for this species of mycobacteria. Samples from this tissue sample could be submitted to an external diagnostic laboratory for further

confirmation of the diagnosis or attempts at DNA extraction for sequencing of products such as 16S rRNA or *hsp65* could be attempted to determine the infection status of this sample.

To conclude, this study attempted to develop a qPCR assay optimised for the detection of mycobacterial pathogens of importance in feline FFPE tissue biopsy samples. Extracting sufficient high-quality DNA from these tissue samples remains challenging, but amplification of mycobacterial DNA was achieved in nearly 80% of samples. For samples infected with MTBC pathogens, qPCR returned a result of MTBC infection in 64% of these but discriminating between infection due to *M. bovis* or *M. microti* was more complicated due to poor performance of the RD1-targeted primer/probe set. Novel primer/probe sets were designed for the detection of *M. avium*, *M. lepraemurium*, and *M. malmoense*; the most encouraging of these appears to be the primer/probe against the *M. lepraemurium*-specific gene MLM_3300. This qPCR may provide a useful diagnostic tool to identify such infections, particularly following an IGRA diagnosis of NTM infection with a PPDA-biased response. Further work is required to optimise the qPCR protocol and DNA extraction techniques, as well as to fully validate primer/probe sets against gDNA from a wider range of mycobacterial species and non-mycobacterial organisms in order to provide this as a sole diagnostic test.

# Chapter 8: General Discussion

Mycobacterial infections have plagued human and animal populations for millennia (Rothschild *et al.*, 2001, Spigelman *et al.*, 2015) and they continue to cause a substantial burden of disease to this present day (World Health Organization, 2021). Advances in our understanding of these infections in humans have led to improved diagnostics, treatments, and outcomes (Nahid *et al.*, 2006, Raju *et al.*, 2016, Aamir *et al.*, 2018), but there are still challenges to be overcome such as effectively treating cases of multidrug- and extremely-drug resistant TB and preventing further infections if the aim of eliminating the TB epidemic by 2030 is to be achieved (Chakaya *et al.*, 2020). Accomplishing this will also depend on addressing the fact that some of these infections are zoonotic in nature, namely due to *M. bovis*, and that a 'One Health' approach is required to truly eliminate TB in humans (Olea-Popelka *et al.*, 2017, Dean *et al.*, 2018). It is not surprising that most research into mycobacterial infections in veterinary species focus on *M. bovis* and cattle (Vordermeier *et al.*, 2001, Buddle *et al.*, 2003, Hope *et al.*, 2005, Waters *et al.*, 2006a, Maggioli *et al.*, 2015, Salguero *et al.*, 2017, Villarreal-Ramos *et al.*, 2018), given the enormous economic costs associated with controlling bTB and that the bovine-human interface represents a major route of infection of people with *M. bovis* (Olea-Popelka *et al.*, 2017, Dean *et al.*, 2018). What is of note is the comparative lack of research into mycobacterial infections of companion animal species.

Reports of mycobacterial infections in companion animal species, particularly cats, go back as far as the late 19$^{th}$ century (Jensen, 1891) with case reports published in human medical journals (Lawford and Neame, 1923), such was the acknowledgement that these infections were of both veterinary and human medical importance. Experimental research demonstrated that cats infected with *M. bovis* developed clinical signs and succumbed to their infection, whereas *M. tuberculosis* infection did not result in disease, suggesting that cats truly are resistant to this pathogen (Francis, 1958). However, it is highly unlikely that such experimental procedures would now be possible. Following the routine pasteurisation of cows' milk, reports of feline mycobacteriosis declined and subsequently it became something of a forgotten and neglected disease. In fact, feline TB due to infection with *M. bovis* was considered to be no longer of importance given that no such infections had been detected in GB following the introduction of bTB eradication schemes in the 1950's (Smith, 1964). Valuable knowledge of how to recognise, diagnose, and treat such cases would have been gradually lost from the forethoughts of veterinarians given the extremely low prevalence of feline mycobacteriosis, and further research into these infections would not be deemed of significance for either human or animal health. Cases attributed as FLS from western Canada were described in the 1980's (McIntosh, 1982), and research had attempted to experimentally transmit infection with *M. lepraemurium* and an unidentified non-cultivable mycobacterial species between cats and rodents with limited success (Schiefer and Middleton, 1983), but otherwise publications on feline mycobacteriosis were mostly sporadic and broadly limited to case reports (Huitema and Jaartsveld, 1967, Snider *et al.*, 1971, Jordan *et al.*, 1994, Malik *et al.*, 1994). This somewhat changed, in the UK at least, when 19 cases of feline TB attributed to a pathogen with intermediate characteristics between *M. bovis* and *M. tuberculosis* were described (Gunn-Moore *et al.*, 1996). Retrospectively, these were most likely cases of *M. microti* infection, which

had been infrequently identified as a cause of disease in cats (Huitema and van Vloten, 1960, Huitema and Jaartsveld, 1967).

Since the publication by Gunn-Moore *et al.* in 1996, there has been an increase in reports of both feline tuberculous and non-tuberculous infections in GB, covering the molecular epidemiology of *M. microti* infections (Smith *et al.*, 2009), the histopathological findings of feline mycobacterial lesions (Kipar *et al.*, 2003, Gunn-Moore *et al.*, 2011b), an estimate on the incidence of feline mycobacteriosis (Gunn-Moore *et al.*, 2013), and the results of specialist mycobacterial culture as well as treatment outcomes (Gunn-Moore *et al.*, 2011a, Gunn-Moore *et al.*, 2011b). This recognition of the importance of mycobacterial infections in cats, in particular cases of disease due to *M. bovis*, was reflected by renewed research and the subsequent development of a new diagnostic test for feline mycobacteriosis, namely the IGRA (Rhodes *et al.*, 2008a, Rhodes *et al.*, 2008b). In the subsequent years, feline TB has gained further importance in the veterinary and public awareness, following a localised cluster of cases of *M. bovis* infection which resulted in the genotypically-confirmed transmission of mycobacteria from cats to humans (Roberts *et al.*, 2014, O'Connor *et al.*, 2019) and an outbreak of *M. bovis* in predominantly indoor-only, pedigree cats fed a commercial raw food diet (O'Halloran *et al.*, 2019, O'Halloran *et al.*, 2020). Cases of feline TB due to both *M. bovis* and *M. microti* have also been identified and reported with increasing frequency in continental Europe (Rüfenacht *et al.*, 2011, Michelet *et al.*, 2015, Cerná *et al.*, 2019, Peterhans *et al.*, 2020), while there have been many reports of feline NTM disease from Australia and North America (Fyfe *et al.*, 2008, Horne and Kunkle, 2009, Malik *et al.*, 2013, O'Brien *et al.*, 2017a, O'Brien *et al.*, 2017b, O'Brien *et al.*, 2017c, Munro *et al.*, 2021).

Despite the increased awareness of feline mycobacterial infections, it remains challenging to diagnose such cases, hence it is of paramount importance for both feline and human health that this addressed. This thesis aimed to expand on the range of diagnostic tests available for feline mycobacteriosis as well as improve currently available methodologies. This encompassed (i) the histopathological and immunohistochemical description of feline mycobacterial lesions and the identification of features that could suggest infection with either *M. bovis* or *M. microti*, (ii) improving the test performance and understanding of the IGRA in its role to diagnose feline mycobacterial infections, its use for follow-up testing in cases of mycobacteriosis that have been treated, and its potential application as an *ante mortem* diagnostic for use in other felid species, (iii) developing a novel ELISA for the diagnosis of feline TB and (iv) developing a novel qPCR for use on FFPE tissues.

Mycobacteriosis is often not considered as a differential diagnosis in cats until the point of histopathological examination of tissue biopsy samples of lesions. Given the importance of histopathology for the diagnosis of feline mycobacteriosis, this thesis incorporated two studies into the histopathological and immunohistochemical features of feline mycobacterial lesions; one of these studies investigated whether such features could discern between infection with the causes of TB in cats, *M. bovis* and *M. microti* (Chapter 3) (Mitchell *et al.*, 2021b), and the other described the histopathological appearance of ocular lesions and considered whether the cat could serve as a model for cases of human OTB (Chapter 2) (Mitchell *et al.*, 2020). A key finding was that (muco)cutaneous

and lymph node lesions from cats with *M. bovis* infection differed in their histopathological appearance compared to lesions from cats infected with *M. microti*. Feline *M. bovis* lesions displayed features similar to those seen in other species such as cattle (Wangoo *et al.*, 2005), with a central core of caseous necrosis, a cellular layer predominantly comprised of epithelioid macrophages, and surrounded by collagen fibres forming a capsule; such lesions were termed 'organised' based on the conserved structure of the granuloma. In contrast, lesions from cats infected with *M. microti* showed an 'atypical' appearance, as these lesions mostly consisted of small clusters of epithelioid macrophages surrounded by a thin fibrous capsule. This description of feline *M. microti* lesions was consistent with that reported by others (Peterhans *et al.*, 2020). This difference in lesion appearance may facilitate an earlier index of suspicion of infection with either pathogen, especially in cases of suspected mycobacteriosis from areas that are considered 'edge' areas for bTB, as well as highlighting whether a case of feline mycobacterial infection may be associated with a higher or lower risk of zoonotic transmission. These findings appeared to translate well to cases of ocular mycobacteriosis, with 'atypical' lesions seen in cases of *M. microti* infection whereas 'organised' lesions were reported in cases of infection with *M. bovis*. It was also noted that many lesions did not fit either of these descriptions and were termed 'unstructured'. Other key findings from this ocular study were that lesions were typically identified in the choroid and retina, and choroidal lesions had the highest assigned inflammation score and were associated with the presence of AFB. These features were suggestive of haematogenous dissemination of mycobacteria to the eye, with the subsequent recruitment of inflammatory cells to the choroid and the development of lesions, with progression to the retina and the rest of the uveal tract. There was significant involvement of lymphocytes within choroidal and retinal lesions, including large numbers of B-cells which were not a common feature of (muco)cutaneous and lymph node lesions. This may suggest there are unique immunological features of the eye in its response to infection; this was also reported in a study of ocular presentations of FIP (Ziółkowska *et al.*, 2017). However, the distribution of feline ocular mycobacterial lesions and the presence of AFB in 80% of globes would suggest that the cat may not serve as a suitable model for human OTB, and consequently other species could be investigated for this role, such as cattle. Other important findings from this study were that two cases of ocular mycobacteriosis were diagnosed as infection with *M. lepraemurium*; only one confirmed case of ocular disease due to *M. lepraemurium* has been reported previously (Ghielmetti *et al.*, 2021), and lesions appear to be restricted to the cornea, conjunctiva and sclera. Such lesions may be permissible to localised surgical excision without the requirement for enucleation and antimycobacterial therapy, as this pathogen does not appear to cause disseminated disease (O'Brien *et al.*, 2017c). Finally, cases of TB in both studies presented with variable numbers of AFB, and some of these were graded with a high BI; the presence of numerous AFB has been historically attributed to NTM infection whereas cases of TB have been considered to have few-to-no detectable AFB (O'Halloran *et al.*, 2016, O'Brien *et al.*, 2017a). These studies both present data that contradict this statement, and therefore it is tantamount that the number of AFB present on ZN-staining is not taken to predict infection with either MTBC or NTM pathogens, especially for the suggestion of infection with zoonotic *vs*. non-zoonotic mycobacteria. Following on from this, further studies should look at further feline TB lesions to determine whether the features identified in this thesis are supported by larger studies, and these could be compared with

lesions from cats with NTM infections to determine whether it is possible to differentiate between TB and NTM infections, or between RD1-positive and RD1-negative mycobacteria.

The IGRA has been integral for diagnosing cases of feline mycobacteriosis (O'Halloran *et al.*, 2020, Albuquerque *et al.*, 2021), yet it lacks validity as an approved diagnostic test for such infections (Middlemiss and Clark, 2018). This thesis presents data critically evaluating the performance of the IGRA for the diagnosis of feline mycobacterial infections (Chapter 4) (Mitchell *et al.*, 2021e), as well as reflecting on its utility for monitoring the response to treatment in such cases (Chapter 4) (Mitchell *et al.*, 2021c). A major finding was that current test cut-off values are not sensitive for the diagnosis of *M. bovis* infections in cats; approximately 44% of cats infected with *M. bovis* in this study showed a specific IFNγ response to stimulation with the antigenic cocktail ESAT-6/CFP-10 compared to a reported sensitivity of 80% (Rhodes *et al.*, 2011). This, in conjunction with current test interpretation guidelines, would result in such cases being misreported as infected with *M. microti*, greatly underestimating the zoonotic risk posed from such cases. It was also shown that cats infected with many NTM species can generate a PPDA-biased response rather than just *M. avium*, namely *M. lepraemurium*, *M. malmoense*, and *M. smegmatis*, all of which have differing prognoses and would be treated with different antimicrobial protocols. Statistical analysis of test data recommended a reduction in cut-off values to increase the sensitivity of the IGRA, for the identification of PPD-bias to infer infection with an MTBC or NTM pathogen, and for the detection of a positive ESAT-6/CFP-10 response in *M. bovis*-infected cats; this increased to almost 69%. Data were also interrogated for factors associated with test failure, and it was showed that more tests failed stimulation with the positive mitogen control during spring and summer compared to autumn. This could reflect the effects of temperature, therefore thermoregulated sample delivery should be considered to help address this fact. Further work is required to validate the IGRA, notably to generate specificity data against clinically healthy cats, and proposed new cut-off thresholds should be externally validated against a new cohort of samples with both IGRA and culture and/or PCR-confirmed diagnosis of mycobacteriosis. Another area that requires further investigation but was beyond the scope of this thesis is the role of IGRA testing for clinically healthy animals that have been exposed to mycobacteria. This should be followed up with physical examination and survey imaging to determine whether there is evidence of subclinical disease, and these cats should be followed for a defined period to see if they develop active disease. This would help establish whether latent infection truly occurs in cats, and whether treatment of IGRA-positive, clinically healthy animals should be recommended as there is a current deficit in our knowledge regarding this controversial topic.

The results of IGRA were analysed for cats that had undergone pre- and post-treatment testing, as there is limited data to suggest whether serial testing is of benefit to determine how long cats need to be treated, and whether vets should aim for reversion to IGRA negativity as the end-point of treatment and if this is associated with longer-term freedom from disease. It was shown that 72% of cats remained IGRA positive (persistent positive) at the point where treatment was stopped as clinical resolution of lesions had been achieved; this figure was similar to what has been reported in humans (Clifford *et al.*, 2015), where serial IGRA testing is not recommended. Remaining positive at the end of treatment IGRA was not associated with recurrence of disease, therefore it would appear that IGRA responses cannot

predict longer-term outcomes. One feature that was identified in all cats that had recurrent disease was the presence of an abnormal pulmonic pattern on thoracic imaging during initial diagnostic investigations. Despite the small number of cases in this study, pulmonic pathology may provide a useful indicator for the likelihood of cases that will relapse or have recurrent mycobacterial disease following treatment. Studies with a defined period of follow-up are essential to help determine longer term prognosis in cases of feline mycobacterial infection to help inform owners when they are faced with making the decision to treat their cat for mycobacteriosis.

The IGRA was initially developed as a diagnostic test for bTB in Australia (Wood *et al.*, 1990), and has since been adapted for other species such as the cat, but also for wildlife species (Waters *et al.*, 2008). However, one limitation is the availability of species-specific reagents. An IGRA was developed using lion-specific monoclonal antibodies (Maas *et al.*, 2012), but this has yet to be implemented as a routine diagnostic test, and the generation of panels of species-specific reagents may prove laborious. Therefore, the work presented in this thesis investigated whether anti-cat IFNγ antibodies could detect rIFNγ from a range of felid species (Chapter 5) (Mitchell *et al.*, 2021d), and whether by extension the cat-specific IGRA could prove beneficial for the *ante mortem* diagnosis of mycobacteriosis in other felids. Bioinformatic analysis showed the high degree of IFNγ genetic conservation across felid species, where data were available, and that the 3D conformational structure of felid IFNγ proteins was broadly similar. As such, anti-cat IFNγ antibodies were able to bind to all felid rIFNγ proteins, whereas anti-dog and anti-bovine antibodies showed minimal cross-reactivity with felid proteins. In contrast, anti-bovine IFNγ antibodies detected bovine and canine rIFNγ but did not bind to felid rIFNγ, and anti-dog IFNγ antibodies only detected canine rIFNγ. This work would suggest that anti-cat IFNγ antibodies are suitable for the detection of IFNγ across felid species and that the IGRA may be adaptable for use as a pan-felid IGRA without the requirement of species-specific reagents. This could be of major benefit for testing animals prior to movement between zoological collections, or as part of reintroduction programmes. While it has been shown that these antibodies can bind to rIFNγ from different felid species, the next step would be to test stimulated samples from these species to see if there is a difference between binding patterns with recombinant and native proteins.

This thesis also presents data on two novel diagnostic tests for the diagnosis of feline mycobacteriosis. The first was the development of an ELISA for the detection of antibodies against PPDA, PPDB and ESAT-6/CFP-10 (Chapter 6) and the second was a qPCR designed to identify and discriminate between infections with MTBC pathogens, as well as species-specific detection of *M. avium*, *M. lepraemurium*, and *M. malmoense* in FFPE tissues (Chapter 7). Tests of humoral immunity for the diagnosis of TB in humans are controversial given their poor sensitivity and specificity (Steingart *et al.*, 2011), but they have some benefits in veterinary medicine, in particular for mycobacterial infections in wildlife species where the use of rapid-tests allow for a result within as little as 15 minutes (Lyashchenko *et al.*, 2008). There are few studies investigating the use of antibody-detection tests for the diagnosis of feline mycobacterial infections, and it would appear that they are of limited benefit and as such are not recommended for use in this species (Cousins and Florisson, 2005). The data presented in this thesis demonstrated the development of a comparative PPD ELISA for the diagnosis of MTBC infection in

cats, which had a sensitivity of 40.7% and 100% specificity. While the sensitivity of the ELISA was poor compared to currently available diagnostic tests such as the IGRA, it was notable that PPDB-biased antibody responses were identified in cats that had failed or were negative on IGRA testing. This would suggest that an antibody ELISA may be a useful adjunctive diagnostic in cases where tests of CMI were negative or have failed. Cats that had a failing IGRA result with lack of stimulation to the positive mitogen control may represent a population of animals that have entered a state of anergy and have converted to a Th2 humoral response. The relationship between mycobacterial infection and changes in the host immune response has been demonstrated in cattle (Ritacco *et al.*, 1991), and while this has not been shown in cats it can be hypothesised that there are similarities in the immune response given the greater sensitivity of IGRA compared to this antibody test, and the predominance of T-cells associated with feline mycobacterial lesions on histopathology compared to B-cells (Chapter 3). Testing for mycobacteria-specific antibodies has been performed on stimulated cell supernatants (Raqib *et al.*, 2003) and these appear promising for the diagnosis of TB in some populations of humans, and given that similar stimulated cell supernatants are generated as part of the IGRA methodology, these could be tested to see if antibodies can be detected in supernatants and how such responses compare to antibody detection in peripheral blood samples. Additionally, antibody testing against defined antigens such as MPB83 should be investigated, as this has been suggested as a major immunogenic protein in cases of *M. bovis* infection in cattle (McNair *et al.*, 2001, Wiker, 2009), although when rapid tests incorporating this antigen have been used in cases of feline mycobacteriosis the sensitivity has been poor for cases of *M. microti* infection (Rhodes *et al.*, 2011). Proteomic analysis of *M. microti* culture filtrates may be beneficial to identify major antigenic proteins that would improve the sensitivity of antibody tests to this pathogen (Cho *et al.*, 2009, Infantes-Lorenzo *et al.*, 2017), and given the poor performance of humoral tests for responses against ESAT-6/CFP-10 alternative antigens should be investigated to allow for the differentiation of MTBC pathogens.

Molecular diagnostics are being used more frequently in cases of feline mycobacteriosis, and present an attractive, rapid means of diagnosing infections and can provide a greater level of species-specific pathogen detection compared to immunodiagnostics, as well as being able to identify cases of infection with organisms that cannot be cultivated in the laboratory. However, molecular tests may fail when used on tissues such as FFPE biopsies, because it can be challenging to extract sufficient high-quality DNA from these samples for PCR testing due to the fragmentation of nucleic acids resulting from formalin fixation, but these samples are frequently available and have untapped potential for diagnosing feline mycobacterial infections. Subsequently, this thesis aimed to develop a qPCR test for use on FFPE tissues for the diagnosis of such infections. Four extraction methods were tested to select an appropriate method to obtain sufficient DNA from FFPE tissues, and mycobacterial-specific DNA was amplified from almost 80% of test samples. The qPCR assay showed a sensitivity of 64% for the detection of IS*6110*, an MTBC-specific marker, in cases of MTBC infection, and while two-thirds of samples from cats infected with *M. microti* were positive to an RD12 primer/probe, only 30% of *M. bovis* samples were positive for an RD1 primer/probe. For the detection of *M. avium* and *M. lepraemurium* novel primer/probe sets were designed against genes that are specific for each species. The results of

qPCR showed two-thirds of samples with an initial diagnosis of *M. avium* were positive to the *M. avium*-specific marker IS*1311*; the remaining two samples were positive on the *M. lepraemurium*-specific primer/probe, potentially indicating these samples were originally misdiagnosed. Three of the four samples from cats infected with *M. lepraemurium* were positive to the *M. lepraemurium*-specific primer/probe designed against the gene MLM_3300 (Benjak *et al.*, 2017). This assay showed reasonable sensitivity for *M. microti*, *M. avium*, and *M. lepraemurium* infections and at this stage may be beneficial for the diagnosis of such infections where a PPDB-biased, ESAT-6/CFP-10 negative result is recorded on IGRA to discern between *M. microti* (RD12-positive) and *M. bovis* (RD12-negative), or between two species of mycobacteria that can both give PPDA-biased responses (*M. avium* and *M. lepraemurium*). Further work is required to improve the sensitivity for RD1 detection, which is essential for the diagnosis of *M. bovis* as this is the only region of the genome present in this organism which is absent in *M. microti*, as well as to improve the extraction of DNA from FFPE samples. This could be achieved through automated methods, which are increasingly used in diagnostic laboratories as they show better performance compared to manual methods for DNA extraction. This assay is also limited as to the species of mycobacteria that can be detected and would either require the identification of more species-specific genetic sequences for other mycobacteria of significance that can infect cats in order to design primers and probes against these targets, or combining qPCR with conventional PCR and sequencing of genes with hypervariable regions such as *hsp65* or 16S rRNA. This assay could also be adapted to include testing to detect antimicrobial resistance, such as mutations in *rpoB* conferring resistance to rifampicin (Kocagoz *et al.*, 2005), as there is limited data available concerning antimicrobial resistance in feline mycobacterial isolates. Given the concerns surrounding drug-resistant strains of mycobacteria, it would be prudent to demonstrate that cases of feline mycobacteriosis, and in particular cases of feline TB, are sensitive to drugs such as rifampicin given we need to safeguard its use for cases of human TB (World Health Organization, 2019a).

In conclusion, the data presented in this thesis significantly contributes to our understanding of feline mycobacterial infections, in particular pertaining to the diagnosis of such infections and how these can be adapted to improve test performance. This in turn results in more accurate tests, reducing the risk of misdiagnosis, and this allows for the implementation of appropriate treatment protocols to improve case outcomes. Improving and expanding our diagnostic capabilities for feline mycobacterial infections will also help to safeguard human health, as it will allow for the more rapid diagnosis of infection with *M. bovis*, a pathogen with proven cat-to-human zoonotic potential. Further studies should focus on the host immune response to mycobacterial infection, as this will allow for more specific diagnostic investigation and understanding of parallels between infection with different species of mycobacteria, as well as allowing for comparisons with infection in other species. Altogether, this thesis demonstrates that while challenging, improvements can be made to the current diagnostic landscape for cases of feline mycobacteriosis, which would be to the benefit of both cats and humans alike.

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