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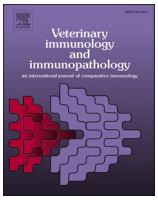

Development of an enzyme-linked immunosorbent assay for the diagnosis of feline tuberculosis

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ABSTRACT

Cases of feline tuberculosis (TB) can be challenging to diagnose. Currently, this is achieved through a combination of mycobacterial culture, polymerase chain reaction (PCR), or interferon-gamma release assay (IGRA); however, these each have limitations. There is limited data regarding the use of humoral immunodiagnostics for TB in cats. Therefore, we sought to develop an enzyme-linked immunosorbent assay (ELISA) to further facilitate the diagnosis of feline TB. A comparative PPD (purified protein derivative) antibody ELISA was optimised for use on serum and plasma, and was tested against samples from 14 cats with culture-confirmed TB and 24 uninfected controls. Selection of an appropriate positive cut-off value based on receiver-operator characteristic curve analysis gave test sensitivity of 64.3 % and specificity of 100 %. When tested on further samples from cats with strongly suspected mycobacteriosis, 32.9 % (23/70) were antibody positive. Notably, positive results were recorded in cats that failed to respond to the IGRA, and in one PCR and IGRA negative cat. No positive responses were identified in cats with non-tuberculous mycobacterial infections, or with non-mycobacterial diseases (n = 12). Therefore, antibody-based diagnostics may be useful adjunctive tests for cases of TB missed by the IGRA, helping protect both feline and, in turn, human health.

1. Introduction

Tuberculosis (TB), resulting from infection with *Mycobacterium* (*M.*) *bovis* or *M. microti*, is increasingly recognised as a cause of morbidity in cats in Europe (Gunn-Moore et al., 2011, Michelet et al., 2015, Peterhans et al., 2020), yet it remains challenging to rapidly and accurately diagnose these cases. Specialised mycobacterial culture at an approved laboratory remains the reference diagnostic test for cases of TB in cats in Great Britain (Middlemiss and Clark, 2018), but it can take at least three months to culture *M. microti* (Smith et al., 2009), by which point the clinical condition of the animal may have deteriorated to a point where treatment is no longer feasible. Molecular diagnostics, such as polymerase chain reaction (PCR), are increasingly available (Michelet et al., 2015; O'Halloran et al., 2020), but as for culture, this requires diseased tissue to be submitted for testing and this may not be achievable in all cases. Therefore, the use of immunodiagnostics provides an alternative means for diagnosing cases of feline TB.

An interferon-gamma (IFNγ) release assay (IGRA) that has been adapted for the diagnosis of feline mycobacterial infections (Rhodes et al., 2008), is readily available in Great Britain. This methodology has

Abbreviations: CFP, culture filtrate protein; CI, confidence interval; CV, coefficient of variation; EDTA, ethylene diamine tetra-acetic acid; ELISA, enzyme-linked immunosorbent assay; ESAT, early secreted antigenic target; IFNγ, interferon-gamma; IGRA, interferon-gamma release assay; *M.*, *Mycobacterium*; MTBC, *Mycobacterium tuberculosis*-complex; NTM, non-tuberculous mycobacteria; OD, optical density; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PPD, purified protein derivative; PPDA, purified protein derivative from *Mycobacterium avium*; PPDB, purified protein derivative from *Mycobacterium bovis*; ROC, receiver-operator characteristic; RT, room temperature; SPF, specific pathogen-free; TB, tuberculosis; ZN, Ziehl-Neelsen.

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also been applied elsewhere (Peterhans et al., 2020). However, concerns regarding the sensitivity of the IGRA have been highlighted, particularly for cases of infection with *M. bovis* (Mitchell et al., 2021), which is potentially zoonotic. The IGRA also requires live peripheral blood cells, which can sometimes fail to respond to the positive mitogen control, causing a test quality control fail (Mitchell et al., 2021); this could indicate a state of anergy in these cats (Schwartz, 2003) or poor sample viability. Antibody tests carried out on plasma or serum do not suffer from issues of cell viability and so may prove a useful adjunctive diagnostic approach to identify cases of TB either missed by IGRA (Plackett et al., 1989) or where IGRA is not feasible.

Antibody-based tests for diagnosing cases of human TB are not recommended by the World Health Organization (Steingart et al., 2011), but they have gained greater traction in veterinary medicine (Greenwald et al., 2003, Lyashchenko et al., 2008, Broughan et al., 2013, Waters et al., 2017). In cattle, enzyme-linked immunosorbent assays (ELISAs) for diagnosing TB by detecting antibodies against PPD from *M. bovis* (PPDB) in a singular format, or in a comparative assay by concurrent testing for antibodies against PPD from *M. avium* (PPDA), have been trialled. These assays provide excellent specificity, but have low sensitivity of 62 % for the singular PPDB antibody ELISA and 44 % for the comparative PPD (PPDB-PPDA, ΔPPD) ELISA (Nuñez-Garcia et al., 2018). However, there is limited data for similar assays in cats. Rapid tests have been employed in cases of feline TB resulting from natural or experimental infection with mixed results (Fenton et al., 2010, Rhodes et al., 2011, Ramdas et al., 2015), and they have also been applied to foxhounds (O'Halloran et al., 2018a), but there is minimal data concerning the use of antibody ELISA for diagnosing TB in cats (Kaneene et al., 2002). To date, there are no reported studies employing a ΔPPD ELISA for the diagnosis of feline TB in confirmed cases of disease.

The aim of this study was to develop a ΔPPD ELISA that would detect TB-specific antibodies in cats with culture-confirmed TB. This ELISA was then used to test samples from cats diagnosed with TB by PCR, unconfirmed but strongly suspected mycobacterial disease based on histopathology/cytology with Ziehl-Neelsen (ZN) staining and/or IGRA, and cats infected with non-tuberculous mycobacteria (NTM) or non-mycobacterial disease to determine whether this ELISA could add value to the diagnosis of TB.

2. Materials and methods

2.1. Ethical statement

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

2.2. Sample acquisition and storage

Opportunistically obtained remnant samples (separated serum, heparin plasma, or ethylene diamine tetra-acetic acid [EDTA] plasma) from cats undergoing diagnostic investigation for suspected mycobacterial disease were submitted to the Royal (Dick) School of Veterinary Studies and The Roslin Institute, University of Edinburgh, or Biobest Laboratories Ltd, Scotland. Clinical data provided by the primary veterinary surgeon were collated to provide further information for each sample and case. Heparin plasma samples from specific pathogen-free (SPF) cats at the WALTHAM Petcare Science Institute that had been donated for use in a previous study were also made available (O'Halloran et al., 2018b), in addition to samples from three client-owned cats (two indoor-only cats from one household, and one cat with outdoor access and known hunting behaviour from the second household). All samples had been stored at -80 °C prior to testing. Serum from a blood donor cat, that had been stored at -20°C, was also provided from the Hospital for Small Animals, University of Edinburgh.

2.3. Correlation of optical density values across matrices

To determine the effect of matrix on optical density (OD) values, cats with paired serum, heparin plasma, and EDTA plasma samples were identified. Samples were available from ten cats, with comparisons made between serum *vs.* EDTA plasma (n = 8), serum *vs.* heparin plasma (n = 5), and EDTA plasma *vs.* serum plasma (n = 5). Spearman's rank correlation coefficient was calculated for paired OD values between matrices. To determine the degree of bias between OD values from paired samples of different matrices, Bland-Altman analysis was performed. Statistical analyses were conducted using GraphPad 9.2.0 (GraphPad Software, San Diego, California, USA).

2.4. Sample cohorts

Samples were only included if the cat had not received prior treatment with an antimycobacterial protocol of 'triple therapy', consisting of rifampicin, a macrolide/azalide, and a fluoroquinolone (O'Halloran and Gunn-Moore, 2017). Samples were divided into the following cohorts (Supplemental Table S1):

Group 1: culture-confirmed cases of TB (*M. bovis* = 11, *M. microti* = 3).

Group 2: uninfected negative control cats (SPF cats = 20, uninfected pet cats = 3, blood donor cat = 1).

Group 3: PCR-only diagnosed cases of TB (unclassified *M. tuberculosis*-complex [MTBC] = 10, *M. microti* = 2, *M. bovis* = 1).

Group 4: unconfirmed but strongly suspected cases of mycobacteriosis (4.1: ZN-positive histopathology and/or cytology = 28 [Fig. 1]; 4.2: clinical signs consistent with mycobacteriosis, supportive histopathology and/or cytology but without positive ZN staining, and/or a positive response to antimycobacterial therapy post-sampling = 42).

Group 5: non-tuberculous diseased cats (5.1: culture- and/or PCR-diagnosed NTM-infected cats = 5 [*M. avium* = 2, *M. kansasii* = 1, *M. lepraemurium* = 1, *M. smegmatis* = 1]; 5.2: non-mycobacterial disease cats based on review of the supporting clinical data = 7).

2.5. ELISA protocol

Nunc MaxiSorp™ 96-well plates (Thermo Fisher Scientific, Waltham, Massachusetts, USA) were coated with 50 μL/well of matched PPDB and PPDA (3000 IU and 2500 IU respectively; Prionics, Thermo Fisher Scientific) diluted 1:2000 in 0.05 M carbonate-bicarbonate buffer pH 9.6 (Sigma-Aldrich, St Louis, Missouri, USA), and the plates covered and incubated overnight at 4°C. Plates were washed six times with phosphate-buffered saline (PBS) and 0.05 % Tween® 20 (Sigma-Aldrich) 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 200 μL of 20 % soya milk (Alpro, unsweetened, Wevelgem, Belgium) diluted in PBS, covered, and incubated for two hours at room temperature (RT). After washing, samples were diluted 1:50 in 20 % soya/PBS and added at 50 μL/well. Plates were covered and incubated for two hours at RT. Plates were washed, and 50 μL/well of Protein A-Peroxidase (Sigma-Aldrich) diluted 1:20,000 in 5 % soya/PBS was added, the plate covered, and incubated for one hour at RT. After washing, 100 μL of 1-Step™ Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific) was added and the plate allowed to develop for five minutes at RT on a shaker, before stopping the reaction by adding 100 μL/well of 0.5 M sulfuric acid. The OD value for each well was read at 450 nm and 630 nm 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 replicate wells had to be ≤ 20 %. If the CV exceeded 20%, a repeat ELISA was

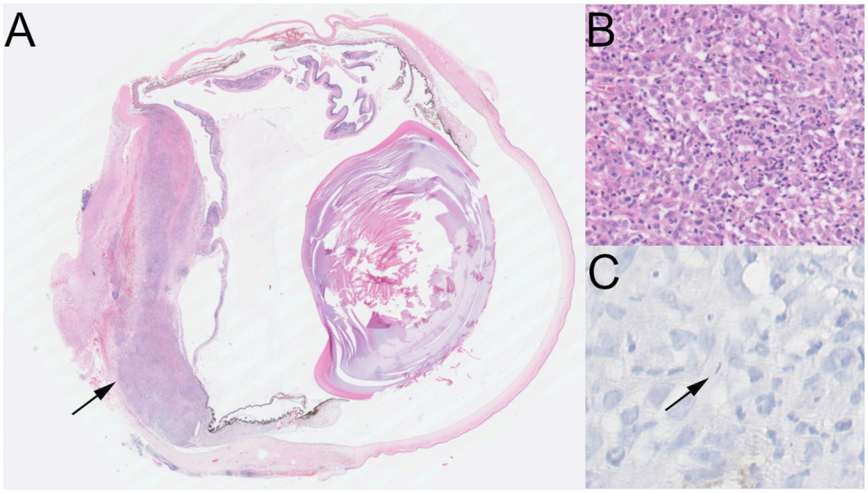

Fig. 1. Enucleated globe from a cat diagnosed with *Mycobacterium bovis* infection by interferon-gamma release assay. Haematoxylin-eosin staining demonstrates marked infiltration and expansion of the choroid, and retinal detachment (black arrow) (A). Higher magnification demonstrates the inflammatory infiltrate to be pyogranulomatous, composed of epithelioid macrophages and neutrophils (B). Ziehl-Neelsen staining identifies a singular acid-fast positive bacillus within the section, morphologically consistent with mycobacteria (black arrow) (C).

performed on that sample.

2.6. Determination of ΔPPD antibody ELISA test sensitivity and specificity

To calculate test sensitivity and specificity, and to establish a positive cut-off OD value for testing further samples, ELISAs were performed on samples from Group 1 (culture-confirmed TB, n = 14) and Group 2 (uninfected negative cohort cats, n = 24). An antibody positive test cut-off value for the ΔPPD ELISA was determined by receiver-operator characteristic (ROC) curve analysis, to maximise test sensitivity and specificity. This was performed using GraphPad 9.2.0 (GraphPad Software, San Diego, California, USA).

2.7. ΔPPD antibody ELISA testing of cases of suspected feline mycobacteriosis

Once a positive cut-off threshold for the ΔPPD antibody ELISA was calculated, samples from Group 3 (PCR-only diagnosed TB, n = 13), Group 4 (strongly suspected mycobacteriosis, n = 70) and Group 5 (non-TB diseased cats, n = 12) were tested to determine the number of antibody-positive cats in each cohort. For cases where an IGRA had been performed, antibody ELISA results were compared to the IGRA to further characterise test performance.

3. Results

3.1. Comparison of OD values across biological matrices

The correlation between ΔPPD OD values on paired samples was assessed across different matrices (serum vs. EDTA plasma [n = 8], serum vs. heparin plasma [n = 5], and EDTA plasma vs. heparin plasma [n = 5]) from cats with confirmed or suspected mycobacterial infection. Spearman's rho values ranged from 0.70 to 1.00 suggesting moderate to perfect positive correlation between all three sample types (Table 1) (Schober et al., 2018). Serum or heparin plasma samples were chosen for further testing as there was the least difference in OD values between these matrices, and these samples are more likely to be readily available for testing in clinical practice (serum) or generated as part of the IGRA methodology (heparin plasma).

3.2. ROC analysis and test performance

A total of 38 serum or plasma samples (14 culture-confirmed cases of TB [Group 1], 24 uninfected cats [Group 2]) were tested by ΔPPD ELISA (Fig. 2A). To establish a cut-off value for ΔPPD positivity, and to calculate test sensitivity and specificity, ROC curve analysis was performed using the OD values for the cats with culture-confirmed TB plotted against the OD values from uninfected cats (Fig. 2B). The area under the curve was 0.87 (95 % confidence interval [CI]: 0.74-1.00), suggesting acceptable to outstanding discrimination of this ELISA to distinguish between culture-confirmed TB-infected and uninfected cats (Hosmer et al., 2013). Selection of a positive cut-off value of 0.13 gave test sensitivity of 64.3 % (95 % confidence interval [CI]: 38.8-83.7 %) *i.e.*, nine out of the 14 culture-confirmed cases of TB were positive on the antibody ELISA, and specificity of 100 % (95 % CI: 86.2-100 %) *i.e.*, no positive antibody results were recorded in the 24 uninfected cats.

Table 1
Results of Spearman's rho analysis for correlation of optical density (OD) values across different matrices for the comparative tuberculin enzyme-linked immunosorbent assay, and Bland-Altman analysis to calculate the average bias between OD values for each matrix comparison.

Matrix comparison	Samples	Spearman's rho	Interpretation	Bias
Serum vs. EDTA plasma	8	0.83	Very strong positive	+0.04 (EDTA plasma)
Serum vs. heparin plasma	5	0.70	Moderative positive	+0.01 (serum)
EDTA plasma vs. heparin plasma	5	1.00	Perfect positive	+0.09 (EDTA plasma)

3.3. Seropositivity in culture and PCR-diagnosed cases of TB

Rates of seropositivity were explored in subgroups of cases of TB diagnosed by culture and/or PCR (Groups 1 and 3, n = 27), applying the cut-off that provided 100 % specificity on ROC curve analysis. Overall, 11/27 (40.7 %) culture and/or PCR TB-positive cats were antibody positive. The test had good ability to distinguish culture-confirmed cases of both *M. bovis* (7/11 cats, 63.6 %) and *M. microti* (2/3 cats), but rates of antibody positivity were lower in cases diagnosed by PCR without culture (Fig. 3). Only 2/13 cats (15.4 %) with a PCR-only diagnosis of TB were antibody positive, one of which was PCR-positive for *M. bovis*. Of the 10 cats with a PCR diagnosis of an unspecified MTBC infection, only one was antibody positive; this cat had an IGRA result suggestive of *M. bovis* infection (PPDB > PPDA, early secreted antigenic target [ESAT]-6/culture filtrate protein [CFP]-10 positive). Of the 13 cats diagnosed with TB by PCR without culture confirmation, nine had an IGRA with a viable result, seven of which suggested MTBC infection (PPDB > PPDA, ± ESAT-6/CFP-10). Based on the supporting clinical and epidemiological data (IGRA results, location, and dietary history)

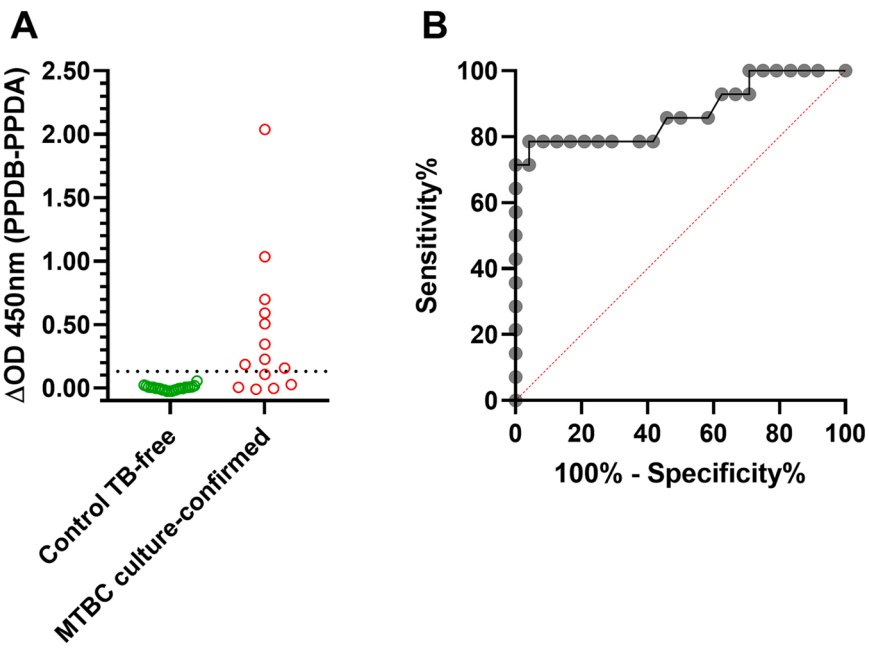

Fig. 2. Results of enzyme-linked immunosorbent assay testing for the ΔPPD response for cats with culture-confirmed tuberculosis (TB), and for uninfected negative control cats. A cut-off threshold for antibody positivity 0.13 (dotted line, A), gave a positive result in 64.3 % of cats with TB. This threshold was calculated from the receiver-operator characteristic curve analysis plotted for cats with TB against uninfected cats (B). OD = optical density. PPDB = purified protein derivative from *Mycobacterium bovis*. PPDA = purified protein derivative from *Mycobacterium avium*. MTBC = *Mycobacterium tuberculosis*-complex.

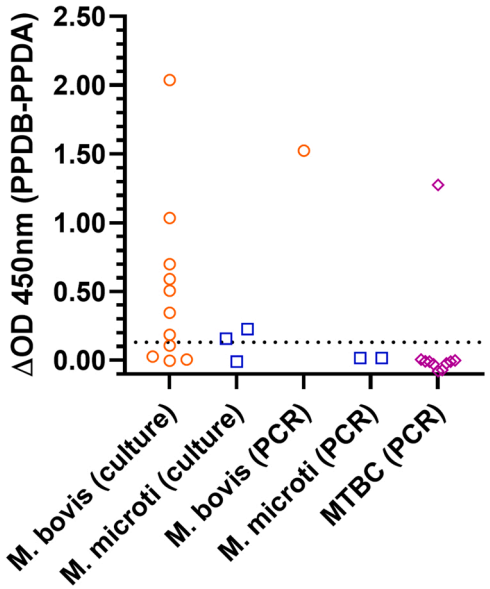

Fig. 3. Results of ΔPPD enzyme-linked immunosorbent assay testing for cats with culture or PCR-confirmed tuberculosis (TB), divided by mycobacterial species and method of diagnosis. The dotted line at 0.13 indicates a positive antibody response. OD = optical density. PPDB = purified protein derivative from *Mycobacterium bovis*. PPDA = purified protein derivative from *Mycobacterium avium*. *M.* = *Mycobacterium*. MTBC = *Mycobacterium tuberculosis*-complex. PCR = polymerase chain reaction.

(Gunn-Moore et al., 2011; O'Halloran et al., 2020; Mitchell et al., 2021), six of the MTBC PCR cats were likely infected with *M. microti*, and the remaining four infected with *M. bovis*.

3.4. Seropositivity in unconfirmed but strongly suspected mycobacteria-infected cats

Overall, 23/70 cats (32.9 %) where mycobacterial disease was suspected but not confirmed by culture or PCR were antibody positive. Of the samples that came from cats with ZN-positive histopathology or cytology, but without culture or PCR confirmation (Group 4.1, n = 28), six cats (21.4 %) were ΔPPD antibody positive (Fig. 4). Three of these cats were IGRA positive (Supplemental Table S1, cases 52, 65 and 66); two had a PPDB-biased, ESAT-6/CFP-10-positive IGRA suggestive of *M. bovis* infection, and one had an IGRA showing equivalent PPDB and PPDA responses, thus a diagnosis of MTBC or NTM infection could not be attributed. The result of this antibody ELISA suggests this cat (case 52) had a MTBC infection. A positive ΔPPD response was recorded in two cats that were not tested by IGRA (Supplemental Table S1, cases 78 and 79), and for one cat that was IGRA negative (Supplemental Table S1, case 77). This latter cat was also mycobacterial PCR negative on the ZN-positive formalin-fixed paraffin-embedded tissue biopsy sample, but had previously consumed a commercial raw food diet associated with *M. bovis* infection in cats (O'Halloran et al., 2019), and responded to treatment with triple therapy. The result of this ELISA (suggesting MTBC infection) in conjunction with the clinical data would support a diagnosis of *M. bovis*.

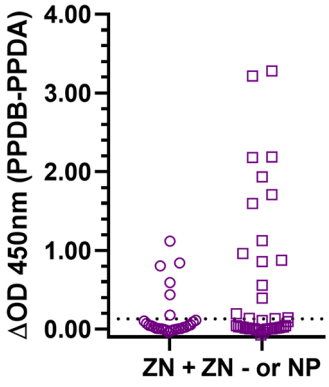

Fig. 4. Results of ΔPPD enzyme-linked immunosorbent assay testing for cats with unconfirmed but strongly suspected mycobacterial infection, divided by results of Ziehl-Neelsen staining, indicating the presence of organisms morphologically consistent with mycobacteria on histopathological or cytological examination. The dotted line at 0.13 indicates a positive antibody response. OD = optical density. PPDB = purified protein derivative from *Mycobacterium bovis*. PPDA = purified protein derivative from *Mycobacterium avium*. ZN = Ziehl-Neelsen. NP = not performed/not provided.

From the cats where mycobacteriosis was strongly suspected, but without a positive ZN result (Group 4.2, n = 42), 17 (40.5 %) were ΔPPD antibody positive (Fig. 4). Thirteen of these antibody-positive cats had a viable positive IGRA result (Supplemental Table S1, cases 87, 88, 90-92, 101, 104, 105, 111-114, and 116), all suggestive of MTBC infection, and seven of these indicated infection with *M. bovis*. One cat was additionally both culture and PCR negative for mycobacterial disease despite a positive IGRA and antibody result (case 88), while a second was negative on specialised culture (case 90). The remaining four antibody positive cats (cases 94, 95, 120, and 121) had failed IGRA quality control thresholds (three positive control fails, one negative control fail) (Mitchell et al., 2021). One of these cases was also negative on mycobacterial PCR (case 94).

3.5. Seropositivity in cats with NTM infections and non-mycobacterial diseases

Samples from five cats diagnosed with NTM infections (Group 5.1) were tested along with those from seven cats where mycobacterial infection was deemed less likely given the supporting clinical data, or an alternative diagnosis was established (Group 5.2). One cat had a PPDA-biased IGRA result but was diagnosed with an *Actinomyces* species infection on PCR (Supplemental Table S1, case 127). The diagnoses of the other cats were idiopathic hypercalcaemia, idiopathic pulmonary fibrosis, pulmonary adenocarcinoma, chronic bronchitis, feline infectious peritonitis, and suspected bacterial bronchopneumonia. These diagnoses were reached by the primary veterinary surgeon during the diagnostic investigation of each case. All 12 cats across both subgroups were negative on the ΔPPD ELISA (Fig. 5).

4. Discussion

The aim of this study was to develop an antibody ELISA for use in cats to diagnose cases of TB. Despite the low sensitivity of this ΔPPD antibody ELISA compared to the IGRA for identifying infections with MTBC pathogens (Mitchell et al., 2021), this small study shows that antibody testing may still play a role in the diagnosis of feline TB.

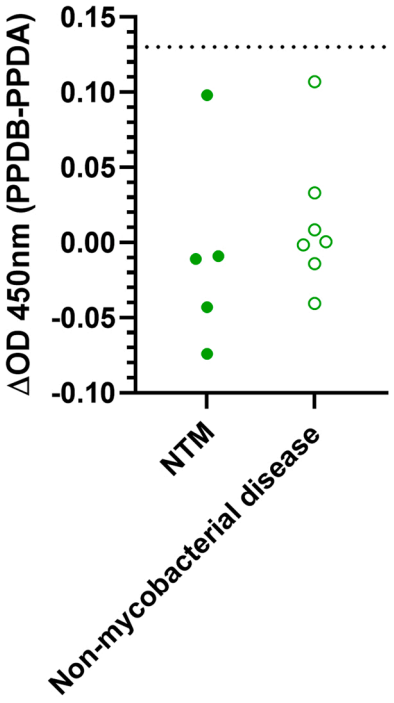

Fig. 5. Results of ΔPPD enzyme-linked immunosorbent assay testing for cats with non-tuberculous mycobacterial disease, or clinically sick cats diagnosed with something other than mycobacteriosis. The dotted line at 0.13 indicates a positive antibody response. OD = optical density. PPDB = purified protein derivative from *Mycobacterium bovis*. PPDA = purified protein dericative from *Mycobacterium avium*. NTM = non-tuberculous mycobacteria.

Analysis of subgroups of cats showed good test sensitivity for cases of MTBC infection diagnosed by culture (64.3 %, 9/14), but poor sensitivity for cases diagnosed by PCR without culture (15.4 %, 2/13). Overall, rates of antibody positivity were higher in cats with culture or PCR-diagnosed *M. bovis* (66.7 %, 8/12) than *M. microti* infection (2/5). Rates of antibody positivity were slightly higher in cats where mycobacterial infection was suspected but not confirmed by culture or PCR (6/28 [21.4 %] cats with ZN-positive lesions, 17/42 [40.5 %] cats without a positive ZN result). Of these 23 antibody-positive cats, 17 had a viable IGRA result, of which 15 (88.2 %) had an IGRA suggesting MTBC infection. One cat had an equivalent PPDA and PPDB IGRA result, while the other antibody-positive cat was negative on IGRA. A further four antibody-positive cats had an IGRA result that failed quality control thresholds. Additionally, no antibody-positive responses were identified in cats with NTM infections or diagnosed with a non-mycobacterial disease. Overall, this suggests reasonable performance of this ΔPPD antibody ELISA for identifying cases of TB, particularly those caused by infection with *M. bovis*.

The traditional paradigm of the immune response in TB infers that as cell-mediated immunity wanes, there is a rising antibody response (Ritacco et al., 1991), and this is associated with progressive pathology and increased rates of recovery of mycobacteria on culture (Vordermeier et al., 2002, Smith et al., 2009). As mycobacterial culture remains the reference diagnostic test for cases of feline TB (Middlemiss and Clark, 2018), these cats were selected as the positive control cohort for ROC curve analysis and determining test sensitivity and specificity. This may explain why rates of antibody positivity were higher in cats with a culture-confirmed diagnosis of TB compared to those diagnosed by PCR; these culture-positive cats have more advanced disease, and as such are more likely to have generated an antibody response detectable by ELISA, whereas cats tested by PCR may have been diagnosed at an earlier stage post-infection and have not yet produced a quantifiable PPDB-specific antibody response.

It was notable that the rate of antibody positivity also differed between culture or PCR-diagnosed cases of *M. bovis* infection (66.7 %) compared to *M. microti* (40 %). The number of culture- or PCR-confirmed cases of *M. microti* infection was low, which is a limitation of the study, but when factoring in the cases diagnosed with MTBC infection on PCR none of those suspected of *M. microti* infection based on supporting clinical data were antibody positive. This discrepancy between antibody positivity rates in cases of *M. bovis* and *M. microti* infection is supported by data from a previous study (Rhodes et al., 2011). This may be attributable to the clinical status of the animals in these studies *i.e.*, those with *M. bovis* infection may have had more advanced disease than those infected with *M. microti*, or it may signify that *M. bovis* generates a more pronounced antibody response compared to *M. microti*. Differences in cytokine concentrations have been reported between cases of feline *M. bovis* and *M. microti* infection (O'Halloran et al., 2018b). Therefore, differences may also occur in the humoral response to infection.

Other important findings from this study include the identification of positive antibody responses in cats that failed IGRA quality controls, as well as those that were negative on culture, PCR, IGRA, or a combination of these tests. Currently, it is recommended to re-sample cats that are IGRA negative, or that have failed quality controls to repeat the IGRA (Mitchell et al., 2021). However, if the positive mitogen control has failed due to T cell anergy, repeat IGRA may also result in test failure. Given that the IGRA is performed on heparinised blood, the plasma harvested when processing the sample provides an additional sample that may be tested in the ΔPPD antibody ELISA, thus allowing for a second immunological test on the same sample, and an additional opportunity to identify infection.

The identification of a positive antibody response in an IGRA-negative cat is important, especially in a test that suggests 100 % specificity. Previously, it had been suggested that a negative IGRA despite ZN-positive staining indicated infection with a non-*M. avium* NTM species (O'Halloran and Gunn-Moore, 2017). However, it has since been shown that negative IGRA results can occur in cases of TB (Mitchell et al., 2021). A concurrent antibody test could facilitate the earlier identification of such cases missed by the IGRA (Watt et al., 2019), allowing for the instigation of appropriate follow-up and treatment protocols for the cat and any affected humans (Gunn-Moore, 2014; O'Connor et al., 2019).

Immunodiagnostic methods for identifying TB in any species can be challenging, given the wide range of mycobacteria that can infect and cause disease in a host. Here we have used a simple comparative PPD protocol that measures MTBC-specific antibody responses above a background of common/environmental, non-MTBC responses. Humoral diagnostic tests for TB in other species have focused on the use of defined proteins or polypeptides (Greenwald et al., 2003, Lyashchenko et al., 2008, Lyashchenko et al., 2021). The ESAT-6/CFP-10 antigenic cocktail can discriminate *M. bovis* infection in the feline IGRA (Mitchell et al., 2021), but previous studies have suggested poor antibody responses to these proteins (Fenton et al., 2010, Rhodes et al., 2011). During this current study, we investigated antibody responses to ESAT-6/CFP-10 to discriminate between infection with *M. bovis* and *M. microti*, but sensitivity was poor at 15.4 % (data not shown).

In cattle, MPB83 and MPB70 have been identified as major immunogenic proteins for the diagnosis of *M. bovis* infection (McNair et al., 2001, Cho et al., 2009, Whelan et al., 2010, Waters et al., 2011). Positive antibody responses to MPB83 have been reported in cats, but the performance for *M. microti*-infected cats was poor compared to those infected with *M. bovis* (Rhodes et al., 2011). It is also possible that NTM could incite responses to this major mycobacterial protein, such as *M. kansasii*, which has been reported in cats (Lee et al., 2017, Černá et al., 2020, Fukano et al., 2021), and so the concern for single-antigen testing in cats is the potential misdiagnosis of this infection as TB.

While these results are promising for the development of an antibody-based diagnostic test for cases of feline TB, there are further factors that could be explored, such as sample quality that might affect test outcomes. For example, haemolysis and freeze-thawing of samples have been investigated for their effects on the ability to detect antibodies against bacterial and viral pathogens in different host species, with variable results (Pinsky et al., 2003, Neumann and Bonistalli, 2009, Boadella and Gortázar, 2011, Kurian et al., 2012, Kragstrup et al., 2013). The samples used in this study were not scored for the degree of haemolysis, nor for the number of freeze-thaw cycles they underwent, so this is something to be aware of in future studies.

One major limitation of this study is the use of the same population for determining test sensitivity and specificity as for performing ROC curve analysis. A positive cut-off value can be selected which will maximise test performance parameters on that specific population, hence it may overestimate the performance of the test once applied to other samples. In this study there were insufficient samples with a culture-confirmed diagnosis of mycobacterial disease, or known non-infected animals, to test as two groups of samples; one for performing ROC curve analysis to select a positive cut-off threshold and a second for using this value to determine test sensitivity and specificity. This could contribute to why the proportion of antibody-positive cats where mycobacterial disease was strongly suspected but not confirmed was lower (21.4-40.5 %) than the stated sensitivity (64.3 %). Alternatively, samples from confirmed positive and negative cases could be requested and ELISA performed on these to assess the external validity of the pre-determined positive test threshold and to determine sensitivity and specificity on an 'unbiased' population.

Antibody-based tests have traditionally been overlooked for the diagnosis of TB (Ritacco et al., 1991, Pollock et al., 2005). 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). The current study presents a simple ΔPPD ELISA method for the diagnosis of TB in cats, with the promise of excellent specificity (100 %) and reasonable sensitivity (64.3 %), crucially identifying infected cats missed by other tests, or where no other test was available. Therefore, while a negative result would not rule out TB, a positive result would be highly supportive of such a diagnosis. This ELISA could prove useful as an adjunctive test for cats 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, which would benefit both feline and human health.

5. Conclusions

In conclusion, this study presents a ΔPPD ELISA that could be used to facilitate the diagnosis of TB in cats. Further work is required to validate this assay with the use of prospectively acquired samples, and to determine inter-operator reliability. Crucially, this ELISA can identify TB-specific antibodies in cats that are negative when tested with currently available diagnostic tests, allowing for timely implementation of treatment.

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Declarations of Competing Interest

None.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetimm.2022.110538.

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