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 293T 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.

Keywords: tuberculosis; interferon-gamma; immunological toolbox; felid; wildlife

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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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; Miller *et al.*, 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 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.

Materials and Methods

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.

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 2µg of pUC57-Amp plasmid containing the gene insert was re-suspended in 10µL of UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, Waltham, Massachusetts, USA); 0.5µg of reconstituted plasmid was added to 50µL of *E. coli*-DH5α and kept on ice for 20 minutes, followed by heat shocking at 42°C for 45 seconds before being put back on ice for two minutes. This was added to 500µL of S.O.C. Medium (Invitrogen) and incubated at 37°C for 60 minutes. Following this, 20µL of this suspension was streaked onto LB plates supplemented with 100µg/mL ampicillin and incubated overnight at 37°C. A single colony was inoculated into 200mL LB broth with 100µg/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, 1µg of plasmid DNA (pUC57-Amp and pFUSE-hIgG1-Fc1) was added to 1µL EcoRI (New England Biolabs, Ipswich, Massachusetts, USA), 1µL XhoI (New England Biolabs), 2µL 10x CutSmart Buffer (New England Biolabs), made up to 20µL in UltraPure™ DNase/RNase-Free Distilled Water and left at 37°C for 60 minutes. To each digest 3.3µL of 6X Gel Loading Dye (Thermo Fisher Scientific) was added, and then run on a 1% agarose gel for 60 minutes at 130V. 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 1µL digested plasmid DNA, 1µL 10X T4 DNA Ligase Reaction

Buffer (New England Biolabs), 1µL T4 DNA Ligase (New England Biolabs), 1µL or 3µL of IFNγ gene DNA and made up to 10µL 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 10mL LB broth with 1:4,000 zeocin and incubated at 37°C overnight with shaking. Stocks of transformed bacteria were made by adding 500µL of bacterial culture to 500µL 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 0.5µg of plasmid DNA, 1µL of restriction enzymes (0.5µL per enzyme as appropriate [NotI and either NcoI or AflII (New England Biolabs)], 1µL of 10x CutSmart Buffer and 0.1µL of bovine serum albumin were added and made up to 10µL in UltraPure™ DNase/RNase-Free Distilled Water. These were left for 60 minutes at 37°C before addition of 1.67µL of 6X Gel Loading Dye and then run on a 1% agarose gel for 60 minutes at 130V.

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 100ng/µL in 5µL UltraPure™ DNase/RNase-Free Distilled Water, with 5µL of forward or reverse primers at 5µM (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 10mL LB broth, then added to 200mL 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 400µL ZymoPURE Elution Buffer, the EndoZero Spin Column was placed in a sterile 1.5mL Eppendorf and the elute added to the column, left for two minutes, centrifuged at 10,000 x *g* for 60 seconds and the concentration and quality of the eluted DNA quantified as above. Samples were stored at -20°C.

Transfection of HEK 293T cells

A volume of 1mL of HEK 293T cells at 1 x 10^7 cells/mL was added to 9mL 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 1mL in supplemented media before being added to 30mL 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 hours at 37°C, at which point cells had reached approximately 80% confluence.

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

For each construct, 2µg of DNA was diluted in 50µL Opti-MEM™ (Gibco) plus 1µL Lipofectamine 2000 (Invitrogen) and incubated for five minutes at room temperature, before addition of 50µL Opti-MEM™. From each well, 1.2mL of media was removed and 100µL of Opti-MEM™/Lipofectamine 2000 was added. The plate was gently agitated for 60 seconds and then incubated at 37°C for four hours. A total of 1mL of supplemented media was added to each well and the cells incubated at 37°C for four days. For each construct transfection of HEK 293T 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.

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.

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 (Figure 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 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 (Figure 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 293T 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 (Figure 3A). Two-fold serial dilutions of the supernatant from 1:12.5 to 1:32,000, was performed. The concentration of 'Cat' rIFNγ exceeded 62.5pg/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 (Figure 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 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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Declarations of Interest

None

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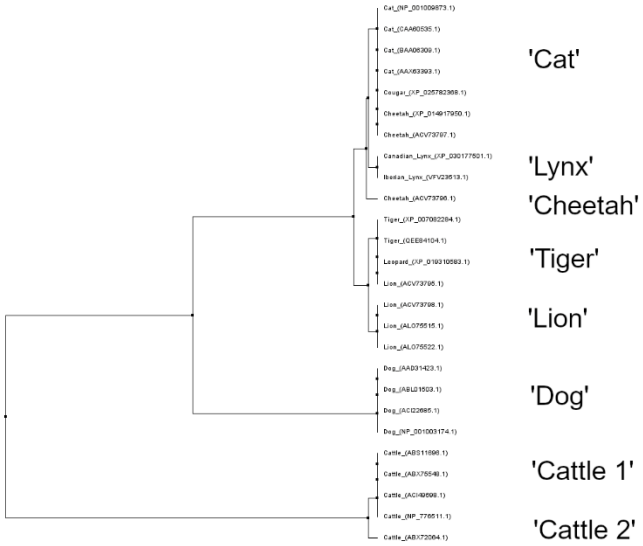
Figure Legends

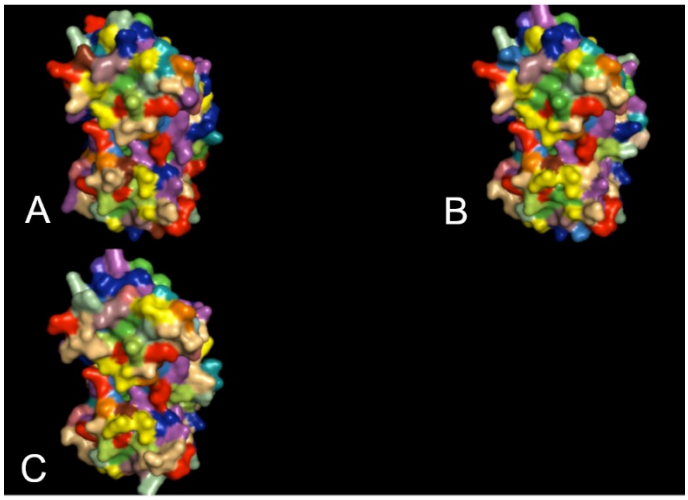
Figure 1: Average distance percentage-identity tree of interferon-gamma amino acid sequences from eight different Felidae species, the dog and from cattle. Five unique interferon-gamma proteins were identified from the seventeen Felidae sequences, categorised as ‘Cat’ (domestic cat, cougar and cheetah),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

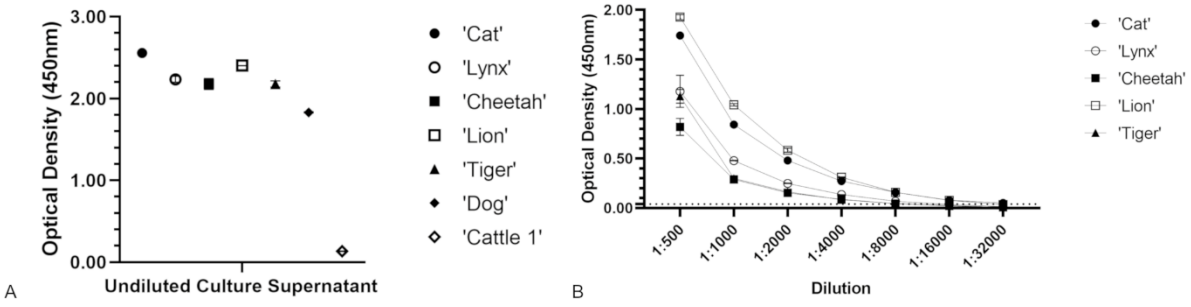
Figure 2: Three-dimensional models of (A) ‘Cattle 1’ (B) ‘Dog’ and (C) ‘Cat’ interferon-gamma proteins predicted by SwissModel and visualised on ‘show surface’ setting using PyMOL to show differences

in the surface structure. Each unique amino acid was assigned an individual colour to highlight differences in the sequence.

Figure 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 (62.5pg/mL). Data are plotted as average OD with error bars to show the range.







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	'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

509

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

512