Agarose gel serum protein electrophoresis in cats with and without lymphoma and preliminary results of tandem mass fingerprinting analysis

Short title: SPE and proteomics in cats with and without lymphoma

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Abstract

Background: Serum electrophoretic profiles in cats are poorly characterized with respect to the protein components of the globulin fractions, and interpretation of the electrophoretograms has routinely been done in ignorance of the identity of the proteins found within each fraction.

Objectives: To compare the protein fractions from serum protein electrophoresis (SPE) in healthy cats and those with lymphoma and to confirm some component proteins in the major fractions after feline SPE, using tandem mass fingerprinting analysis (TMFA).

Methods: Total protein was measured and agarose gel SPE performed on blood collected from 14 healthy cats and 14 with lymphoma. The absolute protein concentration within each fraction was compared between the two groups. Bands corresponding to the SPE fractions were excised from two controls and a lymphoma cat and analysed by liquid chromatography coupled to mass spectrometry. Results were compared to sequences in the NCBI protein database.

Results: Median albumin concentrations were significantly decreased in lymphoma cats and median beta globulin concentrations were elevated. Narrow electrophoretic spikes were present in the beta/gamma fraction in 3 lymphoma cats. Following TMFA, multiple proteins were identified from each fraction and their mobility agreed with results from previous studies generated using alternative techniques. Inter–alpha (globulin) inhibitor 4 was identified in feline serum for the first time.

Conclusions: Cats with lymphoma had lower median albumin and higher beta globulin concentrations than healthy cats. Despite the limitations of 1D agarose gel SPE, TMFA provided preliminary data to confirm the protein components of the various fractions.

Introduction

Serum protein electrophoresis (SPE) on agarose gels has been a technique used in veterinary clinical pathology for several decades for the characterization of serum protein into its main fractions and can provide valuable information in the diagnosis of disease in animals. Most reports in cats regarding SPE are focused on infectious diseases such as Feline Infectious Peritonitis (FIP),[1,2,3,4] Feline Immunodeficiency Virus (FIV) and Feline Leukemia Virus (FeLV).[5] In clinical feline medicine it may also be used in the investigation of hyperproteinemias to differentiate monoclonal and polyclonal gammopathies.[6,7] In humans, characteristic electrophoresis patterns have been found for a variety of conditions including acute inflammation, chronic inflammation and malignant tumors.[8]

The protein fractions of serum are defined by the electrophoretic separation into albumin, which has the highest anodal mobility and the $\alpha-1$, $\alpha-2$, $\beta-1$, $\beta-2$ and γ globulin fractions in order of decreasing anodal mobility. In the serum of normal cats, the globulin fractions have been subdivided further into $\alpha-1$a, $\alpha-1$b, $\alpha-2$a, $\alpha-2$b, $\beta-1$ and $\beta-2$ globulins by investigators using high resolution agarose electrophoresis systems,[9,10] however, this is not routinely performed in diagnostic laboratories. Although the method has been used for many years, the identity of the proteins which comprise the globulin fractions in cats has not been extensively investigated and interpretation of the results of feline serum electrophoretograms has largely been in ignorance of the identity of the proteins found within each fraction. Immunoelectrophoresis of the plasma/serum of healthy cats has identified the location of some, but not all of the plasma/serum proteins on the electrophoretic profile.[11,12] This technique is limited by the availability of appropriate species-specific antibodies. It is generally assumed that on SPE, the serum proteins in other mammals will behave similarly to those in human serum, where this technique has been characterized more fully.[13,14]

There has recently been a rapid development in the proteomic techniques which seek to identify proteins following separation from a complex mixture. This process involves the use of specific

77 protein cleaving agents, usually trypsin, to generate a set of peptides that can be characterized
78 by mass spectrometry. Separation of peptides by liquid chromatography, prior to mass
79 spectrometry and tandem mass spectrometry can enable both amino acid composition and
80 sequence to be inferred for many peptides. Matching of this data to in silico generated peptide
81 and peptide fragmentation databases can allow identification of proteins of interest, providing that
82 genome data is available. This tandem mass fingerprinting analysis (TMFA) approach can be
83 used to characterize proteins separated by 1-Dimensional polyacrylamide gel electrophoresis
84 (PAGE) and is often able to resolve components of bands that are detected by this technique.[15,16]
85 2-Dimensional PAGE allows even better separation of the individual proteins, resulting in more
86 precise determination of the components in each feature (spot) after TMFA, however currently,
87 neither technique is routinely used in clinical laboratories. 1-Dimensional agarose gel
88 electrophoresis (the standard method of analysis for clinical samples with suspected
89 dysproteinemia) results in less complete separation of the proteins hence each band is likely to
90 contain a mixture of proteins. However, agarose gel electrophoresis does have some advantages
91 such as reduced loss of highly charged or hydrophobic proteins which can occur during
92 isoelectric focusing and therefore does have a valid role in proteomic analysis. Furthermore
93 TMFA has recently been used to identify a prominent α-globulin peak on the SPE profile of
94 birds.[17]
95
96 Lymphoma is the most common hemopoietic tumor in cats.[18] SPE has been used to identify
97 monoclonal gammopathies in cats with lymphoma.[19,20] It is likely that other abnormalities in the
98 electrophoretic profile of cats with lymphoma occur, possibly due to changes in acute phase
99 protein (APP) concentrations such as alpha 1-acid glycoprotein (AGP),[21,22] however, no
100 characteristic pattern has been described.
101
102 This study was designed to analyze the protein fractions from SPE in healthy cats and those with
103 lymphoid neoplasia and identify if the globulin fractions are subject to consistent changes in
104 relation to neoplasia. Proteomic analysis was used to identify the component proteins which

make up the major fractions of feline serum following SPE on agarose gels and thus improve the interpretation/utility of feline electrophoretograms. Although bands were excised from both healthy cats, and lymphoma cats to increase the number of proteins identified in the study, the limitations in protein separation with agarose gels outlined above as well as the small number of cases analysed, meant that the comparison between healthy and lymphoma cats was incomplete and only very preliminary.

Materials and methods

Blood samples (3-5ml) were collected from 16 clinically healthy cats and 16 cats with suspected lymphoid neoplasia between November 2006 and February 2009. Written informed consent was obtained from all owners and the study protocol was approved by the Ethics and Welfare committee of the University of Glasgow. The control group consisted of healthy, vaccinated and wormed cats from 2 different first opinion practices in Rome (n=12) which were presented for FeLV and FIV testing prior to routine booster vaccination (against Feline Herpes Virus, Feline Calici Virus, Feline Panleukopenia Virus and FeLV) and from the University of Glasgow Small Animal Hospital feline blood donor registry (n=4) that were used for cross-matching and blood transfusions during the period of the study. All control cats had been vaccinated against the above diseases (except FIV) within the preceding 12 months. They underwent a physical examination, FeLV and FIV testing and biochemical evaluation (the latter performed at the Veterinary Diagnostic Service of the Faculty of Veterinary Medicine, University of Glasgow) and cats with abnormal biochemical results (outwith the laboratory reference range for any analyte) were excluded from the study (n=2). Acute phase proteins (serum amyloid A (SAA), α_1 acid glycoprotein (AGP) and haptoglobin (Hp)) were measured in stored samples using previously established methods, [4, 23, 24] for 11 (Hp and AGP) and 13 (SAA) cats respectively.

Cats with suspected lymphoid neoplasia referred to the Small Animal Hospital, University of Glasgow (U.K.) for confirmation of the diagnosis, staging and treatment were eligible for inclusion in the study (lymphoma group, n=16). Blood was collected prior to treatment. All lymphoma cats

underwent routine clinical staging including a complete blood count, biochemistry profile, FeLV and FIV testing, abdominal ultrasound and thoracic radiography. Acute phase proteins (Hp, AGP, and SAA) were measured on stored samples as above. When appropriate, additional diagnostic investigations were performed at the discretion of the clinician. Samples from cats which had received chemotherapy prior to sampling were excluded from the study (n=2).

Blood was collected into serum tubes (Sarstedt AG & Co, Germany) and allowed to clot at room temperature (20-25°C) before separation of serum by centrifugation (J6-MI Centrifuge, Beckman Coulter, Ireland) at 3000*g* for 5 minutes. The serum samples were stored at -20°C for up to 2 years before analysis when they were gently thawed, homogenized by vortexing and assayed.

The total protein concentration was determined by the biuret method using an automated analyzer (Olympus AU640, Olympus, USA) as previously described.[25] The protein calibrator was prepared from human serum (Olympus System calibrator 66300, Olympus Life Science Research Europa, Germany).

Electrophoresis was performed using an agarose gel electrophoresis system (The Paragon SPE Kit, Beckman Coulter, USA) according to the manufacturer's instructions except that to increase the protein concentration (in order to increased the sensitivity of the TMFA), the samples were not diluted prior to SPE. Four microliters of each serum sample were applied to preformed, numbered sample wells on the agarose gel. Each gel could accommodate up to 10 samples. Control serum Pathonorm™ H (SERO AS, Norway) was included on each gel used. A combination of feline control samples and lymphoma samples were run on each gel. The gels were electrophoresed for 25 minutes at a constant voltage of 100V in 5,5 diethylbarbituric acid (B-2 Barbital Buffer, Beckman Coulter, USA). After electrophoresis, the gels were fixed in acid alcohol (20% acetic acid and 30% methanol, Fisher Scientific UK Ltd, UK) and dried at 37°C for 18-24 hours. Then they were stained in Paragon Blue Stain (0.5% w/v solution) (Beckman Coulter, USA) for 3

minutes, and after destaining in 5% acetic acid solution (Fisher Scientific UK Ltd, UK) and acid alcohol solution, were dried completely.

The stained gels were scanned using a flat bed scanner (UMAX PowerLock III, UMAK UK Ltd, UK) and saved as grayscale TIF files. Computer software (TotalLab Life Science Analysis Essentials, Nonlinear dynamics, UK) was then used to identify the lanes, subtract background and obtain a densitometric trace (electrophoretogram) for each cat (Figure 1). Protein fraction (and sub-fraction) identification and labeling using the software, followed visual examination of each electrophoretogram by three people (MGF, AM, PDE) to reach a consensus on the fraction positions. The relative protein concentration within each fraction was determined by the software as the percentage of optical absorbance of that fraction. The absolute concentration (g/l) of each fraction was then calculated by multiplying the relative protein concentration of each fraction by the total serum protein concentration.

Normality was assessed by visual inspection of box and whisker plots of the data. On this basis, non-parametric tests were used. The median ages, median number of electrophoretic peaks identified, median total protein and median absolute protein fraction concentrations (g/l) as well as APP concentrations were compared between the control cats and the lymphoma cats using a Mann-Whitney U test. Significance was set at $p<0.05$. GraphPad Prism 5 for Windows (GraphPad Software Inc, USA) was used for statistical analyses.

The gels were examined and two control cats with unremarkable electrophoretograms were selected for TMFA. From these cats, bands corresponding to the identified globulin fractions (α–1a, α–1b, α–2a, α–2b, β–1, β–2 and γ) were excised for analysis by proteomics. The albumin fraction was also excised from one cat for further analysis although the main focus of the study was the globulins. In addition, all distinguishable globulin fractions (α–1a, α–1b, α–2, β and γ) were individually excised from one cat with lymphoma. This cat's electrophoretogram was selected as it had no particularly strong bands on visual inspection of the gel, Four other

188 lymphoma cases had bands of strong relative intensities which looked to be of potential clinical
189 significance and these four bands were also excised for proteomic analysis.
190
191 The excised gel bands were washed (with shaking) in 100 mM ammonium bicarbonate (GE
192 Healthcare, UK) for 1 hour at room temperature, followed by a second wash in 50%
193 acetonitrile/100mM ammonium bicarbonate (GE Healthcare, UK). Proteins were reduced with 3
194 mM dithiothreitol in 100mM ammonium bicarbonate (GE Healthcare, UK) for 30 min at 60°C,
195 followed by alkylation with 10 mM iodoacetamide (GE Healthcare, UK) for 30 min in the dark at
196 room temperature. The gel pieces were washed with 50% acetonitrile/100mM ammonium
197 bicarbonate, shaking for 1 hour at room temperature, then dehydrated by incubation with 0.1 mL
198 acetonitrile for 10 min at room temperature. Gel pieces were dried to completion under vacuum,
199 then rehydrated with a sufficient volume of trypsin (Promega sequencing grade, 2 mg/mL in 25
200 mM ammonium bicarbonate (Promega Ltd, UK)) to cover the gel pieces. Digestion was
201 performed at 37°C overnight. The liquid was then transferred to a fresh tube, and gel pieces
202 washed 10 min with a similar volume of 50% acetonitrile. This wash was pooled with the first
203 extract, and the tryptic peptides dried to completion.
204
205 Tryptic peptides were solubilized in 0.5% formic acid (GE Healthcare, UK) and fractionated on a
206 nanoflow high performance liquid chromatography system (FAMOS/Switchos^{TM}/UltiMate, LC
207 Packings, Dionex, USA) before being analysed by electrospray ionisation (ESI) mass
208 spectrometry on a Q-STAR® Pulsar i hybrid MS/MS System (Applied Biosystems Inc, USA).
209 Peptide separation was performed on a Pepmap C18 reversed phase column (LC Packings,
210 Dionex, USA), using a 5 - 85% v/v acetonitrile gradient (in 0.5% v/v formic acid) run over 45
211 minutes. The flow rate was maintained at 0.2 μl/min. Mass spectrometric analysis was performed
212 using a 3 second survey MS scan followed by up to four MS/MS analyses of the most abundant
213 peptides (3 seconds per peak) in Information Dependent Acquisition (IDA) mode, choosing 2+ to
214 4+ ions above threshold of 30 counts, with dynamic exclusion for 120s.
215

Data generated from the Q-STAR® Pulsar i hybrid mass spectrometer was analysed using Analyst QS (v1.1) software (Applied Biosystems Inc, USA) and the automated Mascot Daemon server (v2.1.06) (Matrix Science Ltd, UK). The Mascot search engine was used to compare data against sequences in the current National Center for Biotechnology Information (NCBI) protein database, restricting searches to mammalian sequences. In all cases, variable methionine oxidation was allowed in searches and carbamidomethylation of cysteines was selected as a fixed modification. An MS tolerance of 1.2 Da for MS and 0.4 Da for MS/MS analysis was used. Peptides identified with a MOWSE score greater than 48 ($p<0.05$) were included as this was the identity threshold above which identified parent proteins were considered valid. When proteins matched sequences from multiple species, only the species with the highest combined peptide MOWSE score was included in the table unless a match with a MOWSE score >48 with Felis catus was noted, when this was included instead.

Results

Animals

Serum was collected from 16 control cats based on unremarkable physical examinations but two were subsequently excluded for marked azotemia (n=1) or low total protein and albumin concentrations (n =1). All 14 included control cats were domestic shorthairs (DSH) with a median age of 4 years (age unknown for three cats, range 0.3 to 11 years) and all were FeLV and FIV negative.

Fourteen cats with confirmed lymphoid neoplasia were included in the study (2 additional cats were excluded as they had received chemotherapy prior to sampling). The median age of the lymphoma group was 8 years (range 0.67 to 16 years). Twelve cats were DSH, one was a domestic longhair and one was an oriental shorthair. The cats were presented for a variety of reasons including: lethargy (n=2), inappetance/anorexia (n=2), vomiting (n=3), a palpable abdominal mass (n=4), enlarged peripheral lymph nodes (n=3), dyspnoea (n=4), wheezing (n=1) and coughing (n=1). Five cats had more than one reason for presentation. The cats had had

clinical signs for 1 to 8 weeks prior to presentation (unknown for 3 cats). Details of virus status and the lymphoma site, immunophenotype and method of diagnosis are given in table 1. In all cases, the observed predominant cell type was lymphoblastic not lymphocytic, with coarse, hyperchromatic nuclei, prominent nucleoli, and moderate to high mitotic rate frequently reported. Despite their heterogeneity in anatomical site and clinical presentation, all lymphomas were therefore considered high grade for clinical treatment.

There was no significant difference in the median age of the control and lymphoma groups (p=0.07). The median total protein results for the control group was 74.5g/l (range: 67.0 to 86.0g/l) and was not significantly different from the median total protein for the lymphoma group (74.5g/l, range: 53.0 to 89.0g/l). One cat in the lymphoma group (lymphoma cat 12, figure 2d) was hyperproteinemic (89g/l). APP measurements were obtained for all lymphoma cats and 11 (Hp and AGP) or 13 (SAA) control cats. Median Hp (5.0g/l) and AGP (1.4g/l) were significantly higher in the lymphoma cats (p=0.005, p=0.008) but SAA (median 1.6mg/l) was not significantly different (p=0.189) than in the control group in which the median concentrations were 1.6 g/l for Hp, 0.9 g/l for AGP and 1.2 mg/l for SAA..

Protein electrophoresis

Following densitometer scanning of the electrophoretograms, a minimum of 5 peaks (albumin, α-1, α-2, β and γ) were identified in each cat. In the majority of cats (24/28), α-1 globulins could be further divided into α-1a and -1b fractions. In seven cats, 8 peaks could be identified (albumin, α-1a, α-1b, α-2a, α-2b, β-1, β-2 and γ) (figure 1). There was no significant difference between the median number of peaks identified in the control cats (6 peaks) and the lymphoma cats (7 peaks) (p=0.05). The relative and absolute median values of the protein fractions and the albumin:globulin ratios for the control cats and lymphoma cats are shown (table 2). A statistically significant difference between the lymphoma cats and the control cats was found for the absolute median value of albumin (p=0.046) and β globulin (p=0.018) concentrations. Other comparisons between the groups were not significantly different.

On visual inspection of the gels, there were no consistent differences between lymphoma and control samples but bands with markedly increased intensity were noted in lymphoma cats in α globulin (1 sample), β globulin (2 samples) and γ globulin (1 sample) (bands N, O, P and Q, Figure 2).

Identification of proteins in agarose gel SPE

To identify the proteins present in the globulin fractions, 13 bands were cut from the agarose gels (figures 2a to 2d), 8 from two control cats (A-H) and 5 from a cat with lymphoma (I-M). In each fraction/band, multiple proteins were identified and are listed by name and by NCBI accession number in table 3. The species in which these protein sequences were previously identified is also listed. The percentage of the protein's sequence covered by the identified peptides, Matrix Science MOWSE scores and the number of peptides matched are given as indicators of the closeness of the match. The bands (in figure 2) in which each protein was identified and the type of case from which it was excised (control or lymphoma) is also given. The fractions in which these proteins have been previously reported in the feline and human literature are also listed with their corresponding references.[11,12,13] Eleven proteins were identified from the feline protein database. It can be seen that some proteins were only identified in either the control cats or the lymphoma cat (table 3) although these differences should be interpreted with caution due to the small number of cases analysed. A summary of the proteomic analysis findings with regards to the position of some of the most clinically relevant proteins can be seen in figure 1.

Proteomic analysis of the 4 additional bands of high relative intensity in the lymphoma group (bands N, O, P and Q, figure 2, table 4) showed that.band N (α2 globulin) from cat 4 contained several proteins including haptoglobin and an isoform of ceruloplasmin (acute phase proteins). Bands P (β2 globulin) and Q (γ globulin) contained various immunoglobulins and examination of the electrophoretograms (figure 3a and 3b) revealed narrow spikes in these regions suggestive of monoclonal or oligoclonal gammopathies.[26] Hemoglobin proteins were identified from band O

299 (lymphoma cat 10) suggesting the sample was hemolysed. This sample was slightly red-tinged
300 on gross appearance.
301
302 **Discussion**
303 *Serum protein electrophoresis*
304 This study compared serum protein electrophoretic patterns from a group of normal cats to those
305 from a group of untreated cats diagnosed with lymphoma. No consistent electrophoretic pattern of
306 globulins was found in the cats with lymphoma but the lymphoma population studied was
307 heterogeneous and so in retrospect, this might have been expected. Production of
308 immunoglobulins is not common in feline lymphoma, except for some B cell cases or if secondary
309 infection is present and so this may also have accounted for a lack of consistent differences. The
310 relatively small number of cats may have contributed to insufficient power of the study making
311 consistent changes difficult to determine.
312 Although the total number of identifiable electrophoretic peaks was not significantly different
313 between the two groups, there was a significantly lower median absolute albumin concentration
314 and higher median absolute concentration of β globulins in cats with lymphoma. Albumin is a
315 negative acute phase protein and so the decrease in the lymphoma cats would be consistent with
316 an acute phase response. A significant increase in the α-1 and α-2 globulins (fractions reported to
317 contain positive acute phase proteins in people and cats,[11,12,13]) in the lymphoma cats was not
318 found however, despite the elevated concentration of Hp and AGP in lymphoma cats compared
319 to controls on serum assays. This discrepancy may be because these proteins may not be easily
320 detected by SPE on agarose gels or more likely, because these proteins represent only a small
321 proportion of the overall α-globulins even when their concentration is dramatically increased. An
322 alternative explanation for the lower median serum albumin in the lymphoma cats could be
323 gastrointestinal or renal albumin loss (5 cases had gastrointestinal lymphoma with additional
324 renal lesions in 1 of these cats) or reduced hepatic albumin production. Tests to assess these
325 causes of reduced albumin were not performed in the majority of cases in this study.
326

The significant elevation in β globulins in the lymphoma cats was attributed to two cats in particular which had very high total β globulins, despite total protein being normal. The proteomics results provide information as to the nature of these proteins and in one cat they were the result of hemolysis (cat 10) and in the other cat (cat 14) due to a band containing IgM heavy/constant chains. If the animal with hemolysis is excluded from the analysis (artefactual elevation), the median concentration of β-globulins is still significantly different in the lymphoma cats (P=0.031), however, if both are excluded, the difference is not significant. Further investigation with a larger number of cats would confirm whether a consistent elevation in median β-globulin concentration occurs in cats with lymphoma.

Proteomic analysis

Full proteomic analysis of the SPE fractions of 2 normal cats and one lymphoma cat was carried out to identify some of component proteins which make up the different globulin bands. As expected, multiple proteins were identified from each fraction even in a single cat, [27] (table 3). TMFA enabled us to match peptide fragments to mammalian protein databases. MOWSE scores above 48 indicate matching with greater than 95% confidence; matches above this threshold are listed in table 3 and the great majority of proteins listed are matched at significantly higher confidence. Although many of the matched proteins were encoded by mammalian genomes other than Felis catus, this is likely because the homologous genome sequence is not yet available for cats and our results suggest that the cat homologues of these proteins are indeed present in our samples. The large number of proteins identified in each fraction partly reflects the limited separation achieved on agarose gels and also the large size of some of the excised bands submitted for TMFA. It should be noted that occasionally the peptide fragments match valid sequences in very closely related proteins accounting for some apparent repetition in table 3 eg apolipoprotein A-1 precursor, proapolipoprotein and apoplipoprotein E4. Additionally some poorly characterized proteins are listed eg leucine-rich repeat kinase 1, zinc finger protein 85 although their clinical significance is as yet unknown.

Some of the more clinically relevant proteins identified by TMFA of bands A-M are shown in figure 1C with the corresponding fractions in which they are found. Many of these are serum proteins, with a function in inflammation and the acute phase response eg negative APP such as albumin and serotransferrin (part of transferrin superfamily) and positive APP such as AGP, Hp and ceruloplasmin.[28] Serum measurements of AGP and Hp revealed that both these APP were higher in the lymphoma cat population compared to controls, however, ceruloplasmin was not assayed. Ceruloplasmin and Haptoglobin were also identified in band N (lymphoma cat 4) in the α2 region where they have previously been identified.[11,13] A significant elevation of AGP but not Hp has been reported previously in lymphoma cats, [21] and elevations of AGP, [29, 30] and C-reactive protein CRP, [31, 32] have been reported in dogs with lymphoma. Additional proteins associated with inflammation and immune reactions included complement, immunoglobulins and the soluble form of fibronectin which may be involved in clearance of complement and immune complexes from the circulation.[33] Also identified were various serum enzymes (plasminogen) and enzyme inhibitors (alpha-2 macroglobulin, antithrombin III) involved in control of coagulation and tissue damage. Haemoglobin, hemopexin and albumin which bind iron-containing heme, and iron transporters such as serotransferrin and lactoferrin were also present as were various apolipoproteins (lipid transport proteins). Many of these proteins were also present in the corresponding fractions in the isolated intense bands from lymphoma cats eg alpha-2 macroglobulin in band N (α2 globulin), hemopexin in band O (β globulin).

In addition to these well known serum proteins, there was also the identification of a less well known protein, inter-α (globulin) inhibitor H4 in bands K, F, G, L and M (from both cats with and without lymphoma). This protein is known to be an acute phase protein in pigs,[34,35] with the name of pig-MAP and is also known as plasma kallikrein-sensitive glycoprotein. It has also been identified in humans,[36,37] but has not been described in the cat. Discovery of this previously unsuspected protein from the feline SPE highlights one advantage of proteomics over immunoelectrophoresis since the latter can only be used to look for previously known proteins and only if an appropriate antibody exists.

In most cases, the proteins were identified by proteomic analysis from bands excised from fractions in (or close to) the fractions in which they are expected to be found (table 3).[11,12,13] Many proteins (eg serotransferrin and inter-α (globulin) inhibitor H4) were found to have a wider distribution across the SPE fractions than expected from the literature.[11,12,13,14] This may reflect the presence of different protein isoforms (due to genetic variation or changes in protein glycosylation patterns,[4,14]) or of protein fragments (formed by storage, protein extraction or protein digestion) with different isoelectric points. The presence of albumin in the gamma fraction may be a result of precipitation of this protein at the application site ("X" figure 2).

In each fraction, many proteins were identified in either the control cats or the lymphoma cat (table 3, figure 1), although these differences should not be given much emphasis, considering the preliminary nature of this study and the very few cases analysed. However, lack of detection of a protein in control or lymphoma cats may have been due to a refractory response to trypsin digestion and/or generation of peptides that ionize poorly. In the case of some proteins, the difference may be because they are only synthesized in either normal or lymphoma cats or their synthesis is up or down regulated in disease, eg the acute phase protein AGP, was only identified in the cat with lymphoma. It is also possible that these proteins were present in both affected and non-affected cats but due to the small number of cats in this preliminary comparison, the proteins were not matched in both types of case. Another possibility is that although the proteins were present in both types of cat, the protein migration differed and the proteins were identified in different fractions (eg IgG1 heavy chain).

The TMF analysis was particularly helpful in identifying constituent proteins of highly intense bands in the beta/gamma globulin region of three lymphoma cats (5, 10, 14). In cat 10 (band O) hemoglobin proteins suggested hemolysis was the cause of this electrophoretic peak. In the other two cats (5 and 14, figure 3), immunoglobulins were identified (table 4). The narrow spikes on the electrophoretograms in these two cats are suggestive of monoclonal or oligoclonal

411 gammopathies, rather than polyclonal and further testing with immunoelectrophoresis might have
412 differentiated the type of immunoglobulin.[26] On routine biochemical testing cat 14 had mildly
413 elevated globulin concentration (50g/l, reference range: 27-45g/l) but normal total protein. On
414 electrophoresis, beta globulins (45.5g/l) were highly elevated emphasising the need for SPE
415 analysis in such cases. TMFA identified IgM as the predominant protein in band P (table 4) highly
416 suggestive of a monoclonal gammopathy in the β2 fraction.[19] In cat 5, total proteins and globulins
417 on biochemical analysis and the gamma fraction on electrophoresis were normal. TMFA identified
418 IgG as the strongest matching protein in band Q.
419
420 The inclusion of a relatively small and heterogeneous group of cats with lymphoma with various
421 anatomic forms, immunophenotypes and durations of clinical signs prior to presentation is a
422 limitation of this study. This was likely to have had an effect on the results of comparisons
423 between the SPE profiles of the lymphoma and control cats. If a more homogeneous population
424 were examined, a more consistent pattern might have emerged. The results of the proteomic
425 identification of protein components within the fractions was likely to have been affected by the
426 inclusion of only 3 cats, and potential bias in the way these 3 cases were selected. However this
427 was only a preliminary investigation to illustrate the potential of TMFA in this clinical application
428 and we have successfully demonstrated that this technique can be used to further analyse the
429 constituent proteins of the SPE fractions. A more complete proteomic comparison would have
430 required 2D-PAGE separation followed by TMFA and a larger number of cats.
431
432 To conclude, this study has shown that feline lymphoma patients have lower median albumin
433 concentrations and higher beta globulin concentrations than control cats but identified no
434 consistent elevations in gamma globulins or characteristic electrophoretic patterns. It has
435 established that the protein bands excised from SPE agarose gels can be identified by LC-MS
436 and confirmed previous findings,[11] about the migration pattern of proteins found in the individual
437 fractions following SPE of feline serum on agarose gels. Inter-α (globulin) inhibitor H4, a protein
438 that has not previously been recognized in cats, was identified.

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Figure Legends

Figure 1: Example of the serum protein electrophoresis gel (A) and electrophoretogram (B) for a cat (control cat 3) with 8 identifiable fractions. The y-axis represents the optical density of the band on the gel and the x-axis represents the distance along the gel. The table (C) shows a summary of the position of some of the most clinically relevant proteins as identified by the tandem mass fingerprinting analysis following excision of bands from the electrophoretic gels of control cats 2 and 3 and lymphoma cat 7.

Figure 2: Agarose electrophoresis gels (A to D) from all cats included in the study. Each lane represents serum from a different cat, either control or lymphoma. The final lane on each gel is a control sample (human). ●X represents the application site on the gel. The boxes labeled A to Q represent the areas excised from the gels for peptide fingerprinting analysis and correspond to the 9th column on table 3 (A to M), entitled “Excised band from which found” and the subheadings on table 4. The line marked by an asterisk is thought to be caused by precipitation at the origin on this particular gel. The FeLV and FIV positive cats are identified on the figures. The B or T cell immunophenotype are given for those cats in which this is known or labeled as IPU (immunophenotype unknown) where not done.

Figure 3: Electrophoretograms of lymphoma cats 5 (fig 3A) and 14 (fig 3B) from which bands Q and P were excised showing the narrow-based “spikes”. The y-axis represents the optical density of the band on the gel and the x-axis represents the distance along the gel. The shaded area represents the area excised for proteomic analysis, the results of which are shown in table 4.

Table 1 Clinical details of lymphoma cats

Lymphoma cat number	FeLV status	FIV status	Anatomical site	Method of diagnosis	Immunophenotype
1	negative	negative	gastrointestinal (with renal involvment)	cytology	Unknown
2	negative	negative	gastrointestinal	histopathology	B cell
3	positive	negative	thymic	histopathology	T cell
4	negative	negative	multicentric	histopathology	Unknown
5	negative	negative	extranodal (laryngeal)	histopathology	Unknown
6	negative	negative	multicentric (with thymic involvment)	cytology	Unknown
7	negative	negative	thymic	cytology	Unknown
8	negative	negative	extranodal (pulmonary)	cytology	Unknown
9	negative	positive	extranodal (laryngeal)	cytology	Unknown
10	negative	positive	gastrointestinal	histopathology	Unknown
11	negative	negative	gastrointestinal	histopathology	T cell
12	negative	negative	thymic	cytology	T cell
13	negative	negative	multicentric	histopathology	B cell
14	negative	negative	gastrointestinal	histopathology	Unknown

Table 2: Relative and absolute values of albumin and globulin concentrations in lymphoma and control cats

	Number of Samples		Median Relative Values (%) (range)		Median absolute values (g/l) (range)		
Fractions	Control cats	Lymphoma cats	Control cats	Lymphoma cats	Control cats	Lymphoma cats	p value
Total protein	14	14	NA	NA	74.5 (67.0 – 86.0)	74.5 (53.0 – 89.0)	0.80
Albumin	14	14	40.6 (26.7-48.5)	35.4 (23.3-50.2)	**29.1** (19.8 – 37.3)	**24** (19.3-44.7)	**0.046**
Albumin to globulin ratio	14	14	NA	NA	0.7 (0.4– 0.9)	0.6 (0.3 – 0.7)	0.14
Alpha-1 globulins total	14	14	10.3 (8.6 – 15.1)	10.2 (6.0 – 13.9)	7.7 (6.9 – 10.4)	7.5 (5.0 – 11.1)	0.24
Alpha-1a globulins	12	12	6.3 (3.4 – 9.4)	6.0 (2.4 – 8.1)	4.8 (2.9 – 6.5)	4.2 (2.0 – 6.6)	0.44
Alpha-1b globulins	12	12	3.9 (2.9 – 8.4)	4.3 (3.0 – 6.1)	3.0 (2.5 – 5.8)	3.0 (2.4 – 4.5)	0.95
Alpha-2 globulins total	14	14	18.9 (7.46 – 23.0)	16.7 (10.2 – 25.2)	13.4 (5.3 – 17.8)	12.4 (7.8 – 21.4)	0.32
Alpha-2a globulins	4	6	12.1 (6.7 – 13.1)	15.0 (5.8 – 18.4)	9.1 (5.8 – 9.5)	9.5 (4.6 – 15.7)	0.76
Alpha-2b globulins	4	6	6.7 (6.0 – 10.2)	6.6 (6.2 – 9.9)	5.4 (4.3 – 7.6)	4.6 (3.8 – 7.9)	0.61
Beta globulins total	14	14	10.3 (7.6 – 23.2)	15.0 (11.8 – 45.5)	**7.8** (5.7 – 16.0)	**11.4** (7.3 – 45.5)	**0.02**
Beta-1 globulins	5	12	6.2 (5.5 – 9.5)	8.4 (3.9 – 10.4)	4.7 (4.4 – 6.8)	4.7 (2.5 – 7.7)	0.96
Beta-2 globulins	5	12	7.52 (5.1 – 13.7)	7.0 (4.1 – 40.0)	5.4 (3.7 – 9.5)	5.1 (2.4 – 29.2)	0.43
Gamma globulins	14	14	21.5 (6.9 - 37.4)	19.6 (8.4 – 30.9)	17.2 (4.8 – 27.6)	14.1 (5.0 – 21.9)	0.28

NA – not applicable.
Values shown in bold were statistically significantly different ($p<0.05$)

Table 3: LC-MS identification of proteins from excised albumin and globulin fractions (bands A-M figure 2) following protein electrophoresis of feline serum

Sub-fraction on SPE	Proteins identified	Accession Number	Species of Origin	Sequence coverage (%)	MOWSE Score	Number of peptides matched	Type of case(s) in which peptide found	Excised band in which found	Fraction reported in literature
	Albumin fraction								
NA	**serum albumin precursor**	gi|57977283	**Felis catus**	53	1851	40	control	A	albumin/ α-1 (11, 13)
NA	apolipoprotein A-I	gi|342075	Macaca fascicularis	11	136	3	control	A	albumin/ α-1 (11,13)
	Alpha-1a globulin fraction								
NA	**serum albumin precursor**	gi|57977283	**Felis catus**	30	967	20	control + LSA	B, I	albumin/ α-1 (11, 13)
NA	apolipoprotein A-1	gi|342075	Macaca fascicularis	21	209	7	control + LSA	B, I	albumin/ α-1 (11,13)
NA	**IgG1 heavy chain**	gi|3402543	**Felis catus**	11	110	2	LSA	I	β/ γ (11,12, 13)
NA	inter-alpha (globulin) inhibitor H3*	gi|74011920	Canis lupus familiaris	4	63	3	control	B	NR
	Alpha-1b globulin fraction								
NA	alpha-2-macroglobulin precursor*	gi|73997689	Canis lupus familiaris	4	250	8	control	C	α-2 (11, 12, 13)
NA	**serum albumin precursor**	gi|57977283	**Felis catus**	15	361	8	LSA	J	albumin/ α-1 (11, 13)
NA	apolipoprotein A-I precursor*	gi|73955106	Canis lupus familiaris	19	235	7	control + LSA	C, J	albumin/ α-1 (11,13)
NA	proapolipoprotein	gi|178775	Homo sapiens	22	192	5	LSA	J	NR
NA	apolipoprotein E4	gi|283972743	Panthera tigris	14	76	3	control	C	NR
NA	vitamin D-binding protein*	gi|114594352	Pan troglodytes	8	102	3	LSA	J	NR
NA	**alpha-1 acid glycoprotein**	gi|47825211	**Felis catus**	9	79	3	LSA	J	α-1 (11, 13)
NA	inter-alpha-trypsin inhibitor heavy chain H2	gi|3024062	Mesocricetus auratus	1	60	2	control	C	α-2 (13)
NA	serotransferrin precursor (transferrin) (siderophilin)*	gi|73990142	Canis lupus familiaris	2	55	1	LSA	J	β (11, 12, 13)
NA	protein AMBP (alpha-1-microglobulin) (inter-alpha-trypsin inhibitor light chain)	gi|72507586	Homo sapiens	2	51	1	LSA	J	α-2 (13)
	Alpha-2 globulin fraction								
a + b	alpha-2-macroglobulin precursor*	gi|73997689	Canis lupus familiaris	7	568	10	control + LSA	D, E, K	α-2 (11, 12, 13)
a + b	pregnancy zone protein*	gi|73997687	Canis lupus familiaris	3	234	5	control	D, E	NR
a + b	**haptoglobin**	gi|73990923	**Felis catus**	35	169	4	control + LSA	D, E, K	α-2 (11, 13)
b	**IgG1 heavy chain**	gi|3402543	**Felis catus**	18	153	5	control	E	β/ γ (11,12, 13)
NK	apolipoprotein A-I precursor*	gi|73955106	Canis lupus familiaris	19	152	5	LSA	K	albumin/ α-1 (11,13)
b	hemoglobin subunit beta	gi|122594	Crocuta crocuta	22	147	3	control	E	β (13)
b + NK	complement component C3	gi|47522844	Sus scrofa	2	146	3	control + LSA	E, K	β-2 (11, 13)
b	beta-globin	gi|22874	Gorilla gorilla	19	125	2	control	E	NR
NK	inter-alpha (globulin) inhibitor H4 (plasma kallikrein-sensitive glycoprotein)*	gi|74011918	Canis lupus familiaris	4	112	4	LSA	K	α-2 (13)
b	antithrombin III	gi|179161	Homo sapiens	7	109	2	control	E	α-2 (11)
NK	proapolipoprotein	gi|178775	Homo sapiens	18	104	4	LSA	K	NR
a + b	clusterin precursor	gi|50979240	Canis lupus familiaris	4	90	2	control	D, E	NR
b	Haemoglobin subunit epsilon	gi|122725	Otolemur crassicaudatus	15	88	2	control	E	NR
NK	inter-alpha-trypsin inhibitor family heavy chain-related protein	gi|1483187	Homo sapiens	1	84	2	LSA	K	α-2 (13)
b	alpha-2-HS-glycoprotein precursor (Fetuin-A) (alpha-2-z-globulin) isoform 2	gi|740003450	Canis lupus familiaris	5	83	2	control	E	NR
NK	apolipoprotein B precursor	gi|553189	Homo sapiens	1	79	2	LSA	K	NR
b	apolipoprotein J precursor	gi|178855	Homo sapiens	4	78	2	control	E	NR
b	serotransferrin precursor (transferrin) (siderophilin)*	gi|73990108	Canis lupus familiaris	4	70	2	control	E	β (11, 12, 13)
a	leucine-rich repeat kinase 1*	gi|109082322	Macaca mulatta	1	68	3	control	D	NR
b	A-gamma globin	gi|284005431	Oryctoacus cuniculus	16	64	2	control	E	NR
b	apolipoprotein A-IV*	gi|149716543	Equus caballus	2	64	1	control	E	NR
NK	RIKEN cDNA 1300017J02	gi|18204720	Mus musculus	1	62	1	LSA	K	NR
a + b	ceruloplasmin	gi|1224108	Mus musculus	3	59	3	control	D, E	α-2 (11,13)
b	porcine inhibitor of carbonic anhydrase*	gi|194221612	Equus caballus	1	58	1	control	E	NR
a	kininogen 1*	gi|57109938	Canis lupus familiaris	2	54	1	control	D	NR
a	kininogen 2 isoform 1	gi|41235784	Mus musculus	1	53	1	control	D	NR
NK	melanoma associated antigen (mutated) 1-like 1*	gi|74009138	Canis lupus familiaris	2	53	2	LSA	K	NR
a	murinoglobin 1 precursor	gi|12831225	Rattus norvegicus	1	53	2	control	D	NR
a	zinc finger protein 85 (HPF4, HTF1)*	gi|149626477	Ornithorhynchus anatinus	3	51	2	control	D	NR
b	anionic trypsin-1 precursor	gi|6981420	Rattus norvegicus	8	50	1	control	E	NR

Sub-fraction on SPE	Proteins identified	Accession Number	Species of Origin	Sequence coverage (%)	MOWSE Score	Number of peptides matched	Type of case(s) in which peptide found	Excised band in which found	Fraction reported in literature
	Beta globulin fraction								
1 + 2	serotransferrin precursor (transferrin) (siderophilin)*	gi|73990142	Canis lupus familiaris	16	610	11	control + LSA	F, G, L	β (11, 12, 13)
1 + 2	complement C3 precursor*	gi|194212541	Equus caballus	7	386	10	control	F, G	β-2 (11, 13)
1 + 2	hemopexin*	gi|73988725	Canis lupus familiaris	14	278	6	control + LSA	F, G, L	β (11, 12, 13)
1 + 2	fibronectin*	gi|194211292	Equus caballus	2	231	6	control + LSA	F, G, L	NR
1 + 2	inter-alpha (globulin) inhibitor H4 (plasma kallikrein-sensitive glycoprotein)*	gi|194221223	Equus caballus	5	194	5	control + LSA	F, G, L	α-2 (13)
2	**IgG1 heavy chain**	gi|3402543	**Felis catus**	17	180	4	control	G	β/ γ (11,12, 13)
2 + NK	lactoferrin	gi|186833	Homo sapiens	3	143	4	control + LSA	G, L	NR
1 + 2	lactotransferrin isoform 3*	gi|73985785	Canis lupus familiaris	4	131	4	control	F, G	NR
1	inter-alpha (globulin) inhibitor H1*	gi|194211292	Equus caballus	4	126	4	control	F	NR
1	PK-120 precursor	gi|2739028	Mus musculus	2	117	3	control	F	NR
1	trypsin inhibitor	gi|33985	Homo sapiens	2	101	4	control	F	NR
1 + NK	inter-alpha -trypsin inhibitor family heavy chain-related protein	gi|1483187	Homo sapiens	1	97	2	control + LSA	F, L	α-2 (13)
1	alpha-2 macroglobulin precursor*	gi|73997689	Canis lupus familiaris	2	94	2	control	F	α-2 (11, 12, 13)
2 + NK	complement component C4A	gi|179674	Homo sapiens	1	93	2	control + LSA	G, L	β (11)
2	**immunoglobulin kappa light chain**	gi|6456731	**Felis catus**	8	87	2	control	G	β/ γ (11, 13)
2	**IgM heavy chain**	gi|3402547	**Felis catus**	5	78	3	control	G	β/ γ (11, 13)
NK	proapolipoprotein	gi|178775	Homo sapiens	10	77	2	LSA	L	NR
2	Immunoglobulin heavy chain VHDJ region	gi|38092744	Camelus dromedarius	14	71	1	control	G	NR
1	apolipoprotein B precursor	gi|553189	Homo sapiens	1	71	2	control	F	β (11)
1 + 2	apolipoprotein A-1	gi|3915607	Canis lupus familiaris	6	71	2	control + LSA	F, G, L	albumin/ α-1 (11,13)
1	antithrombin III	gi|179161	Homo sapiens	6	71	2	control	F	α-2 (11)
2	plasminogen	gi|18139619	Canis lupus familiaris	4	70	2	control	G	β (11)
2	**IgG gamma constant chain**	gi|2914001	**Felis catus**	19	70	2	control	G	β/ γ (11,12, 13)
1 + 2	alpha-2 plasmin inhibitor	gi|219408	Homo sapiens	6	66	1	control	F, G	NR
2	sex hormone-binding globulin	gi|38325826	Bos taurus	3	62	1	control	G	NR
2	immunoglobulin lambda-chain	gi|192812	Mus musculus	7	61	1	control	G	NR
NK	CHKSR family member 3	gi|197098578	Pongo abelii	2	58	3	LSA	L	NR
NK	**IgM constant chain**	gi|2914011	**Felis catus**	4	56	1	LSA	L	β/ γ (11, 13)
2	anionic trypsin-1 precursor	gi|6981420	Rattus norvegicus	8	55	1	control	G	NR
NK	hepatocarcinogenesis-specific protein/hemopexin homolog (clone HC34)	gi|1087020	Marmota monax	3	53	1	LSA	L	NR
	Gamma globulin fraction								
NA	**IgG1 heavy chain**	gi|3402543	**Felis catus**	42	524	16	control + LSA	H, M	β/ γ (11,12, 13)
NA	**immunoglobulin kappa light chain**	gi|6456731	**Felis catus**	34	291	6	control + LSA	H, M	NR
NA	**IgM heavy chain**	gi|3402547	**Felis catus**	17	229	5	control + LSA	H, M	β/ γ (11, 13)
NA	immunoglobulin heavy chain variable region	gi|37694585	Homo sapiens	16	169	3	control + LSA	H, M	NR
NA	Ig heavy chain variable region, VH3 family	gi|33319108	Homo sapiens	24	107	3	control + LSA	H, M	NR
NA	immunoglobulin heavy chain VHDJ region	gi|38093044	Camelus dromedarius	16	84	1	control + LSA	H, M	NR
NA	immunoglobulin lambda chain	gi|192812	Mus musculus	7	81	1	control + LSA	H, M	NR
NA	NUAK family, SNF1-like kinase, 2*	gi|291402539	Oryctolagus cuniculus	1	80	2	control	H	NR
NA	**immunoglobulin epsilon heavy chain constant region**	gi|7262603	**Felis catus**	3	78	2	control + LSA	H, M	NR
NA	**albumin**	gi|309262111	**Felis catus**	2	68	2	control + LSA	H, M	albumin/ α-1 (11, 13)
NA	inter-alpha (globulin) inhibitor H4 (plasma kallikrein sensitive glycoprotein)*	gi|126336622	Monodelphis domestica	2	68	2	LSA	M	α-2 (13)
NA	complement factor H precursor (H factor 1) isoform 2	gi|74005944	Canis lupus familiaris	1	67	1	control	H	NR
NA	serotransferrin precursor (transferrin) (siderophilin)*	gi|73990142	Canis lupus familiaris	2	59	1	control + LSA	H, M	β (11, 12, 13)
NA	immunoglobulin lambda-like polypeptide 1 precursor (immunoglobulin-related 14	gi|73995675	Canis lupus familiaris	22	57	2	control	H	NR
NA	immunoglobulin V lambda/J lambda light chain	gi|6643739	Homo sapiens	17	49	2	LSA	M	NR

* NCBI record for these proteins are predicted from the genomic sequence
proteins in bold text are those identified in Felis catus
NA Not applicable
NK sub-fraction not known
LSA Lymphoma
NR migration not reported in human or feline literature

Table 4: LC-MS identification of proteins from specific excised isolated bands (bands N to Q, figure 2) following protein electrophoresis of feline serum

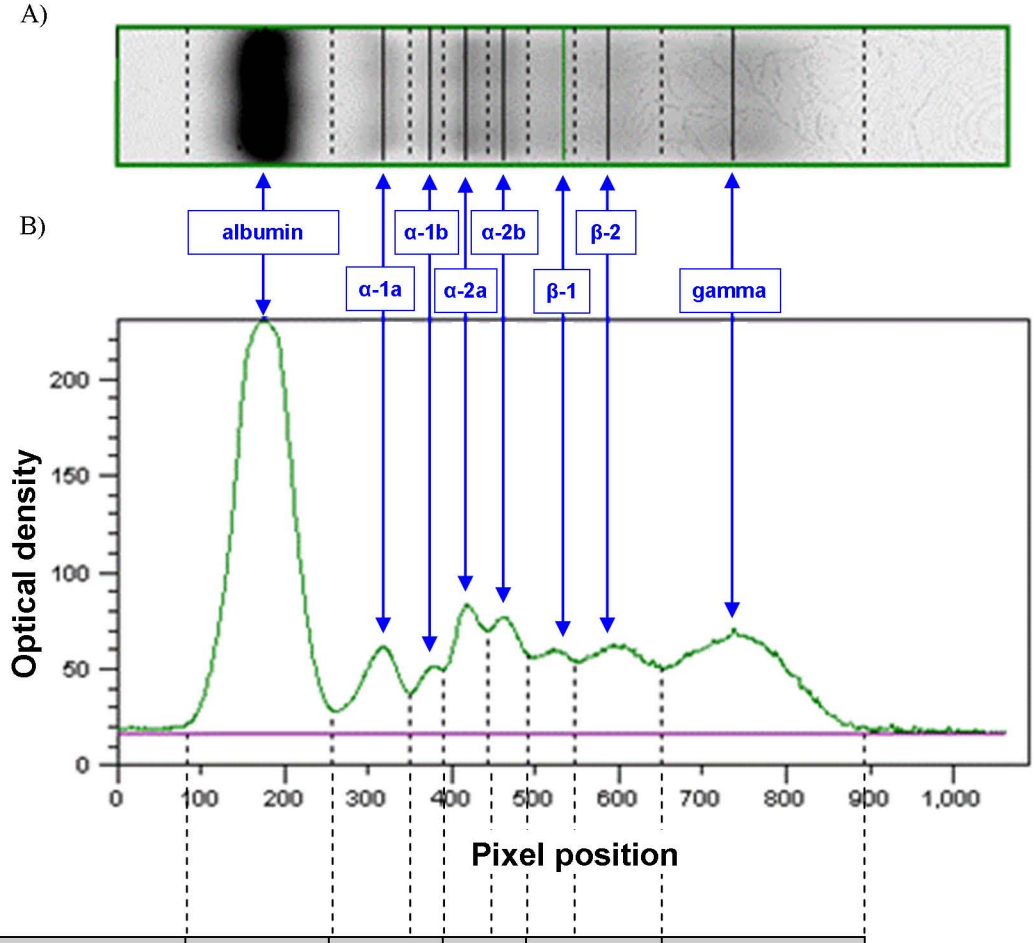
Fraction on SPE	Proteins identified	Species of Origin	Accession Number	Sequence coverage (%)	MOWSE Score	Number of peptides matched	Protein present in control cats in this fraction?	Protein present in lymphoma cat 7 in this fraction?
	BAND N (figure 2)							
alpha 2a	**haptoglobin**	**Felis catus**	gi|73990923	35	175	6	Yes	Yes
	pregnancy-zone protein*	Canis lupus familiaris	gi|73997687	2	158	4	Yes	No
	ceruloplasmin (ferroxidase) isoform 2*	Macaca mulatta	gi|109048806	4	101	3	Yes	No
	alpha-2 macroglobulin precursor*	Canis lupus Familiaris	gi|73997689	1	97	3	Yes	Yes
	hemoglobin beta chain	Macaca arctoides	gi|86611	23	75	3	No	No
	DEAH (Asp-Glu-Ala-His) box polypeptide 37	Monodelphis domestica	gi|126323968	1	48	2	No	No
	BAND O (figure 2)							
beta	haemoglobin subunit beta-2	panthera pardus saxicolor	gi|55584062	67	420	13	No	No
	haemoglobin subunit beta A/B	**Felis catus**	gi|122608	75	374	13	No	No
	haemoglobin subunit alpha	**Felis catus**	gi|122405	46	220	10	No	No
	beta globin	Orycteropus afer	gi|221381007	14	144	3	No	No
	serotransferrin precursor (transferrin) isoform 1*	Canis lupus Familiaris	gi|73990142	7	116	5	Yes	Yes
	alpha globin chain	Mesocricetus auratus	gi|49421	20	106	4	No	No
	haemoglobin subunit epsilon	Bradypus tridactylus	gi|78099200	21	101	4	No	Yes
	hemopexin*	Canis lupus familiaris	gi|73988725	6	82	2	Yes	Yes
	apolipoprotein A-I	Canis lupus familiaris	gi|3915607	6	58	2	Yes	No
	lactotransferrin isoform 3*	Canis lupus Familiaris	gi|73985785	2	50	2	Yes	No
	BAND P (figure 2)							
beta2	**IgM heavy chain**	**Felis catus**	gi|3402547	45	602	15	Yes	No
	serotransferrin precursor (transferrin) isoform 1*	Canis lupus Familiaris	gi|73990142	7	156	5	Yes	Yes
	immunoglobulin heavy chain variable region	Homo sapiens	gi|118405920	15	73	2	Yes	No
	Ig mu chain C region membrane-bound form	Oryctolagus cuniculus	gi|127511	3	61	2	No	No
	NUAK family, SNF1-like kinase, 2*	Oryctolagus cuniculus	gi|291402539	1	60	2	No	No
	SNF histone linker PHD RING helicase*	Monodelphis domestica	gi|126310663	0	56	2	No	No
	Ig heavy chain variable region, VH3 family	Homo sapiens	gi|33319108	15	50	2	Yes	No
	BAND Q (figure 2)							
gamma	**IgG1 heavy chain**	**Felis catus**	gi|3402543	27	177	6	Yes	Yes
	immunoglobulin heavy chain variable region	Homo sapiens	gi|118405920	15	62	1	Yes	Yes
	IgM heavy chain	**Felis catus**	gi|3402547	7	58	2	Yes	Yes
	titin*	Equus caballus	gi|194222358	0	48	6	No	No

* NCBI record for these proteins are predicted from the genomic sequence
proteins in bold text are those identified in Felis catus

Figure 1

A)

B)



C)

Protein	albumin	α-1		α-2		β		gamma
Albumin	■	■						■
Apolipoprotein	■	■	■	■	■	■	■	
Alpha-2 macroglobulin			■	■	■			
Alpha-1-glycoprotein acid			■					
Haptoglobin				■	■			
Serotransferrin			■		■	■	■	■
Haemoglobin					■			
Complement C3					■	■	■	
Inter-alpha globulin inhibitor H4				■	■	■	■	■
Antithrombin III					■	■		
Ceruloplasmin				■	■			
Hemopexin						■	■	
Fibronectin						■	■	
Lactoferrin						■	■	
Plasminogen							■	
Immunoglobulin G		■			■		■	■
Immunoglobulin M						■	■	■

Figure 2

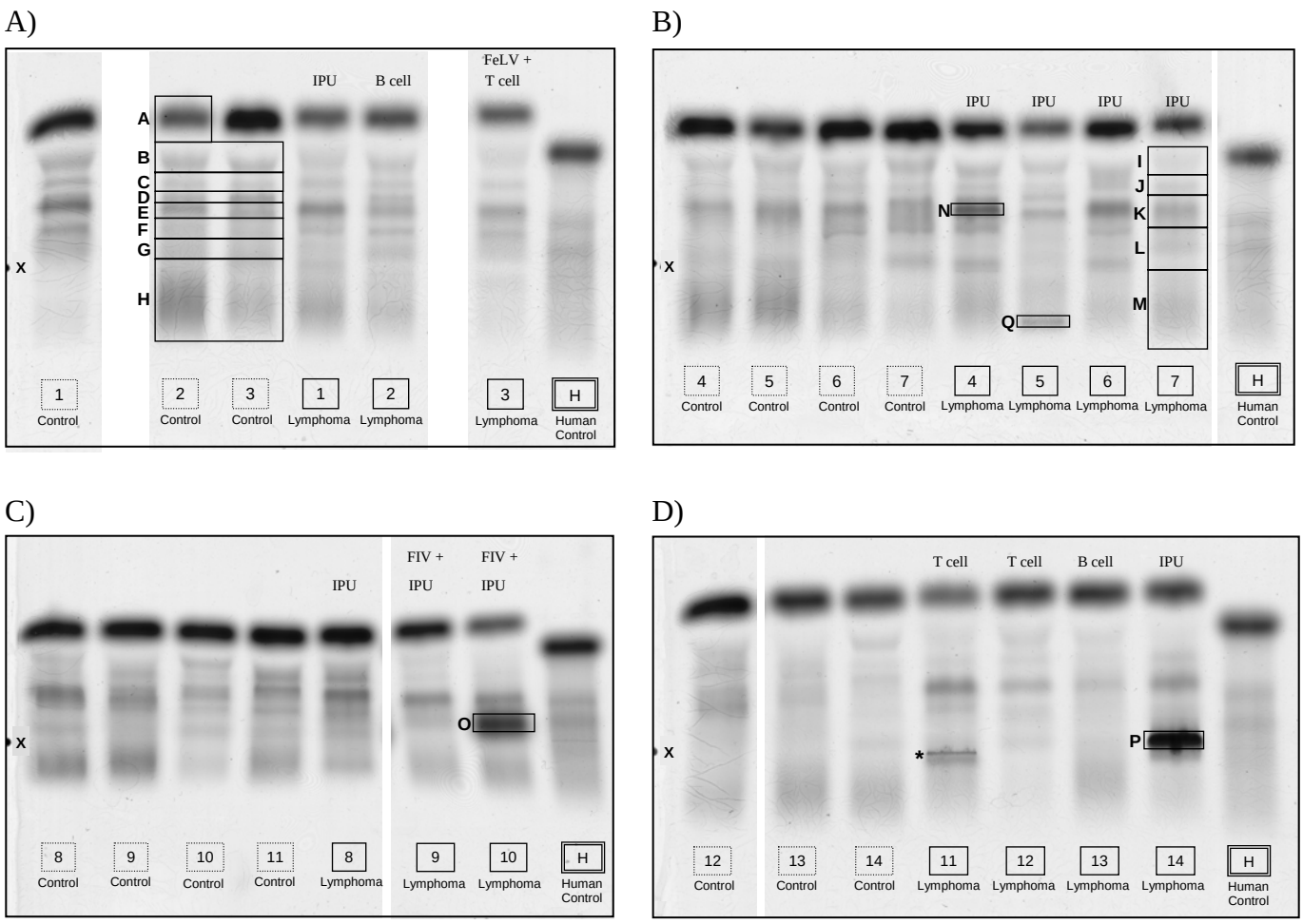
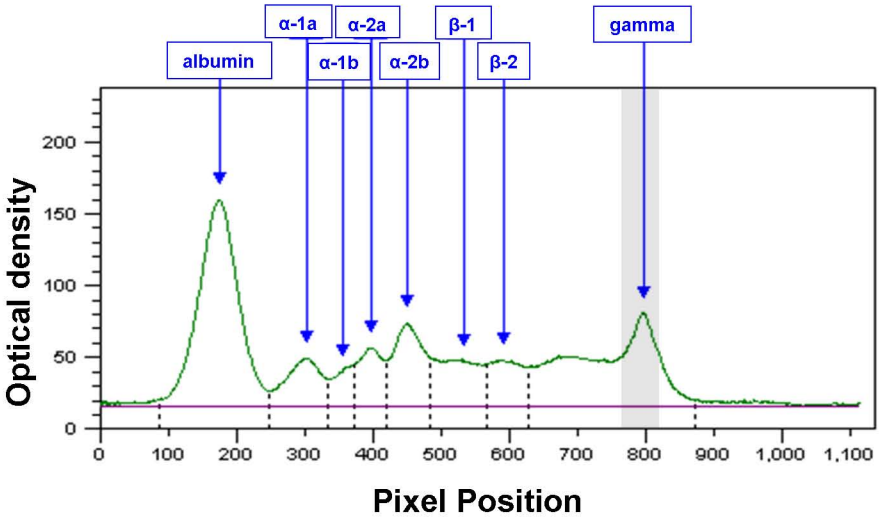
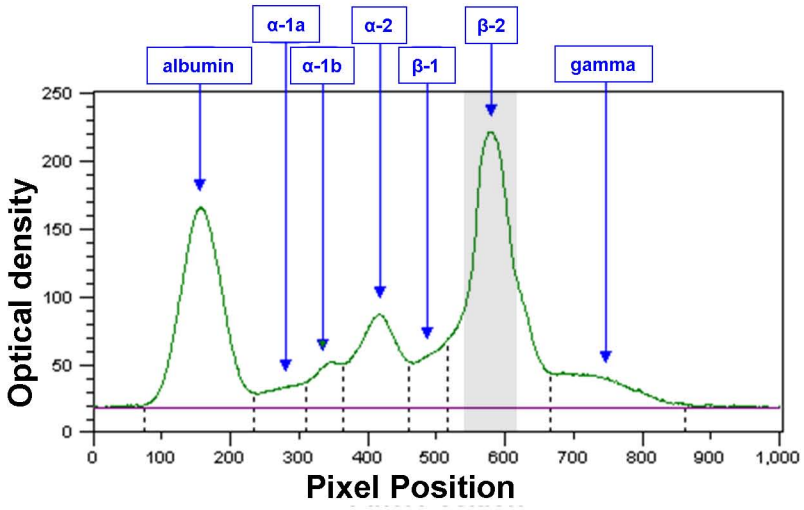

A)
IPU
B cell
FeLV +
T cell
A
B
C
D
E
F
G
H
X
1 Control
2 Control
3 Control
1 Lymphoma
2 Lymphoma
3 Lymphoma
H Human Control
B)
IPU
IPU
IPU
IPU
I
J
K
L
M
N
Q
X
4 Control
5 Control
6 Control
7 Control
4 Lymphoma
5 Lymphoma
6 Lymphoma
7 Lymphoma
H Human Control
C)
IPU
FIV +
IPU
FIV +
IPU
O
X
8 Control
9 Control
10 Control
11 Control
8 Lymphoma
9 Lymphoma
10 Lymphoma
H Human Control
D)
T cell
T cell
B cell
IPU
*
P
X
12 Control
13 Control
14 Control
11 Lymphoma
12 Lymphoma
13 Lymphoma
14 Lymphoma
H Human Control

Figure 3

A)


albumin
α-1a
α-1b
α-2a
α-2b
β-1
β-2
gamma
Optical density
Pixel Position

B)


albumin
α-1a
α-1b
α-2
β-1
β-2
gamma
Optical density
Pixel Position